

Depletion of RhoGDI2 expression inhibits the ability of invasion and migration in pancreatic carcinoma

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Abstract. Rho GDP dissociation inhibitor 2 (RhoGDI2) has been identified as a regulator of tumor metastasis, although its role in tumor progression remains controversial. In this study, we examined the expression of RhoGDI2 in PC tissues and cell lines. To investigate the function of RhoGDI2 in PC cells, RhoGDI2 expression was depleted in PANC-1 and Patu8988 cells by small interfering RNA (siRNA). RhoGDI2 was found to be overexpressed in pancreatic carcinoma (PC) tissues and PC cell lines. Additionally, the results showed that depletion of RhoGDI2 significantly inhibited cell motility and invasion *in vitro*, but did not affect cell proliferation. The clinical study together with the experimental data confirmed that RhoGDI2 modulated the expression of matrix metalloproteinase 2 (MMP2). Taken together, findings of the present study indicated that RhoGDI2 is involved in pancreatic tumor malignancy and metastasis. Thus, RhoGDI2 is a potential target for the gene therapy of PC.

Introduction

Pancreatic carcinoma (PC) is an aggressive and lethal malignancy. The median overall survival is <6 months, with a 5-year survival rate of 5% (1,2). Vascular invasion, perineural invasion and distant metastasis are the critical features in the aggressive phenotype of PC, and contribute to the lost opportunities for surgical resection (3,4). Thus, new therapeutic strategies

should be based on a better understanding of biomarkers and their association with invasion and metastasis.

Rho GTPases, including Rac1, Cdc42 and RhoC, are important regulators of cell migration, cell motility, cell cycle progression and cytoskeleton organization (5,6). The biological activities of Rho GTPases are regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and Rho GDP dissociation inhibitors (RhoGDIs) (7). Rho GDP dissociation inhibitor 2 (RhoGDI2), also known as D4-GDI or LyGDI, belongs to a family of RhoGDIs. RhoGDI2 was originally expressed in hematopoietic cells, predominantly in B and T cells (8,9). However, it has been shown that RhoGDI2 is also abnormally expressed in tumors. The role of RhoGDI2 in tumor progression remains controversial. RhoGDI2 may function as a positive regulator of tumor progression in gastric, colorectal, and breast cancer (10-12). The role of RhoGDI2 as a metastasis suppressor gene was also validated in bladder cancer and Hodgkin's lymphoma (13,14). However, the expression and role of RhoGDI2 in the progression of PC remains to be determined.

In this study, we examined the expression of RhoGDI2 in PC tissues and cell lines. Moreover, using small interfering RNA (siRNA) to silence RhoGDI2 expression, we investigated the effect of RhoGDI2 in the regulation of invasion and metastasis. These findings indicated that RhoGDI2 is a potential target for the gene therapy of PC.

Materials and methods

Clinical samples. Tissue samples from 60 PC patients were collected during surgical resections performed at the First Affiliated Hospital of Soochow University between January 2010 and December 2012. Tumorous and adjacent non-tumorous tissues were frozen immediately after surgical removal in liquid nitrogen and stored at -80°C. The patients did not receive any preoperative chemotherapy, radiotherapy or immunotherapy. The samples were obtained following written patient consent. Study approval was obtained from the local ethics committee of Soochow University.

Cell culture and transfection. Human PC cell lines, PANC-1, SW1990, Patu8988 and BxPC-3 were obtained from the

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Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and maintained in DMEM, supplemented with 10% fetal bovine serum (both from Gibco, Grand Island, NY, USA) and 100 $\mu\text{g}/\text{ml}$ each of penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 at 37°C.

SiRNA transfection. Small hairpin RNA (shRNA) of human RhoGDI2 (NM_001175; GeneBank) transfer vector encoding the green fluorescent protein (GFP) was constructed by GeneChem Co., Ltd. (Shanghai, China). The siRNA sequence targeting RhoGDI2 (siRNA-RhoGDI2) was 5'-GGAAGGUUCUGAAUAUAGA-3', as confirmed by sequencing. The negative control of siRNA (siRNA-NC) was designed and the sequence was without obvious homology to the human gene. Cells (1×10^4 /well) were cultured in 6-well plates overnight and reached 60-70% confluence. To determine the different expressions of RhoGDI2, PANC-1 and Patu8988 cells were transfected with siRNA-RhoGDI2 or siRNA-NC using Lipofectamine 2000 (Invitrogen) as the transfection reagent. Non-transfected cells were used as the blank control (control).

Quantitative PCR (qPCR). Total RNA from tissues and cells was extracted using TRIzol (Invitrogen). Single-strand cDNA for a PCR template was synthesized from 10 μg of total RNA using random primers and M-MLV reverse transcriptase (Takara Bio, Dalian, China). The relative levels of target gene mRNA transcripts to that of the control (β -actin) were determined by qPCR. The primers used were (forward and reverse): 5'-ATGACTGAAAAGCCCCA-3' and 5'-TCATTCGTCCACTCCTT-3' for RhoGDI2 (606 bp); 5'-GTGCTGAAGGACACACTAAAGAAGA-3' and 5'-TTGCCATCCTTCTCAAGTTGTAGG-3' for matrix metalloproteinase 2 (MMP2) (605 bp); 5'-AGCGGGAAATCGTGCGTG-3' and 5'-CAGGGTACATGGTGGTGGCTGCC-3' for β -actin (308 bp). The PCR reactions were 40 cycles (95°C for 15 sec, 62°C for 45 sec, and 72°C for 30 sec). The amplified segments were analyzed by 2.5% agarose gels.

Western blotting. Cells were collected and lysed in lysis buffer on ice. Total proteins were separated by 10% SDS-PAGE and blotted on PVDF membrane. Membranes were blocked with 10% non-fat milk powder at room temperature for 2 h and incubated with primary antibodies: anti-RhoGDI2 antibody (1:200), anti-MMP2 antibody (1:200) (both from Abcam, Cambridge, UK) and anti- β -actin antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. After three washes, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Reactive bands were detected using ECL western blotting detection reagent.

Cell proliferation assay. CCK8 assay was performed to evaluate cell proliferation. Following transfection, the cells were seeded in 96-well plates at 5×10^3 cells/well, followed by the addition of 20 μl CCK8 (Dojindo Laboratories, Kumamoto, Japan) and incubation at 37°C for an additional 2 h. An ultraviolet spectrophotometer (Implen, Munich, Germany) was used to measure the absorbance of each well at 450 nm, and each experiment was performed in triplicate and repeated five times.

In vitro wound scrape assay. Cells from each group were incubated in 6-well plates. A small wound area was made in the confluent monolayer with a 200 μl pipette tip in a lengthwise stripe. The cells were washed twice with PBS and incubated at 37°C. Images were captured at 0 and 48 h. Wound width was measured at a magnification of x100 using a microscope (DM2500, Leica Microsystems, Mannheim, Germany) with a calibrated eyepiece grid (1 mm/100 μm graduation). Ten measurements were determined at random intervals along the wound length.

Invasion assay. Cell invasion was determined using Matrigel-coated invasion Boyden chambers using a Transwell kit, according to the manufacturer's instructions. Briefly, 600 μl DMEM medium containing 10% FBS was added to the bottom chamber. After 12 h of serum starvation, the cells were collected and placed in the upper invasion chamber (1×10^5 cells/well). After incubation at 37°C for 48 h, the non-invasive cells in the upper chamber were removed with a cotton swab. The insert membranes were fixed with methanol for 15 min and stained with 0.1% crystal violet. The stained cells that penetrated through the Matrigel were counted under the inverted microscope.

Immunohistochemistry (IHC). Serial sections (4 μm) were prepared for immunohistological staining. Tissue sections were quenched for endogenous peroxidase with freshly prepared 3% H_2O_2 with 0.1% sodium azide and then placed in an antigen retrieval solution for 15 min. After incubation in a casein block, primary anti-RhoGDI2 monoclonal antibody (1:50; Abcam) was applied to the sections for 1 h at room temperature, followed by incubation with the secondary antibody and extravidin-conjugated horseradish peroxidase. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (>76%). The final score was obtained by the sum of the intensity score and the quantity score. A score ≥ 3 was considered as a positive expression.

Statistical analysis. SPSS version 17.0 was used for statistical analysis. Data were expressed as mean \pm SD. One-way analysis of variance (one-way ANOVA), t-test and Chi-square test were performed for inter-group comparison. $P < 0.05$ was considered statistically significant.

Results

Overexpression of RhoGDI2 protein in PC. In 60 patients who recently underwent surgery for PC, paired tumor samples and non-tumorous tissues were subjected to IHC. The subcellular expression pattern of RhoGDI2 was mainly diffuse cytoplasmic in PC and the non-tumorous tissues were negative or weakly positive for RhoGDI2 (Fig. 1). The positive rate of RhoGDI2 in tumor tissues was 73.3% (44/60) while that in non-tumorous tissues was 41.7% (25/60), showing a significant difference ($\chi^2 = 12.310$, $P = 0.001$).

Correlation between RhoGDI2 protein and clinicopathological characteristics. The relationship between the expression of RhoGDI2 and the clinicopathological characteristics of PC are shown in Table I. RhoGDI2 expression was markedly

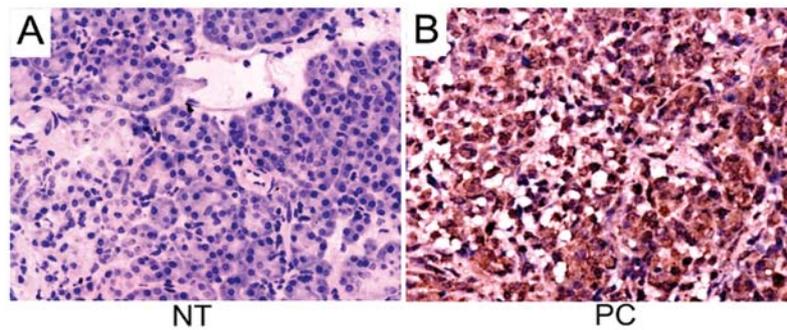


Figure 1. Immunohistochemical staining of Rho GDP dissociation inhibitor 2 (RhoGDI2) protein. (A) Low expression in pancreatic non-tumorous tissues (NT). (B) High expression in pancreatic carcinoma (PC) tissues. Original magnification, x200.

Table I. Relationship between RhoGDI2 expression and clinicopathological characteristics of PC patients.

Characteristics	Cases	RhoGDI2		χ^2	P-value
		Negative	Positive		
Gender					
Male	38	10	28	0.007	1.000
Female	22	6	16		
Age (years)					
≤65	36	13	23	4.105	0.072
>65	24	3	21		
Tumor location					
Head	32	7	25	4.105	0.072
Body and tail	28	9	19		
Tumor size (cm)					
≤2	8	6	2	11.027	0.003 ^a
>2	52	10	42		
Differentiation					
Well	13	6	7	7.172	0.028 ^a
Moderate	15	6	9		
Poor	32	4	28		
Clinical stage					
I	12	8	4	12.273	0.001 ^a
II	48	8	40		
Lymph node metastasis					
Y	41	6	35	9.586	0.004 ^a
N	19	10	9		
Vascular invasion					
Y	18	1	17	5.860	0.023 ^a
N	42	15	27		

RhoGDI2, Rho GDP dissociation inhibitor 2; ^aP<0.05.

correlated with tumor size ($\chi^2=11.027$, P=0.003), differentiation ($\chi^2=7.172$, P=0.028), clinical stage ($\chi^2=12.273$, P=0.001), lymph node metastasis ($\chi^2=9.586$, P=0.004) and vascular invasion ($\chi^2=5.860$, P=0.023), but did not show a statistically significant association with gender ($\chi^2=0.007$, P= 1.000), age ($\chi^2=4.105$, P=0.072) and tumor location ($\chi^2=4.105$, P=0.072).

Depletion of RhoGDI2 by siRNA in PC cells. First, we examined the relative expression of RhoGDI2 at the mRNA and protein levels in the PANC-1, SW1990, Patu8988 and BxPC-3 cell lines, with PANC-1 and Patu8988 cells being selected as a high expression of RhoGDI2 (Fig. 2A and B). To investigate the function of RhoGDI2 in PC cells, we depleted RhoGDI2

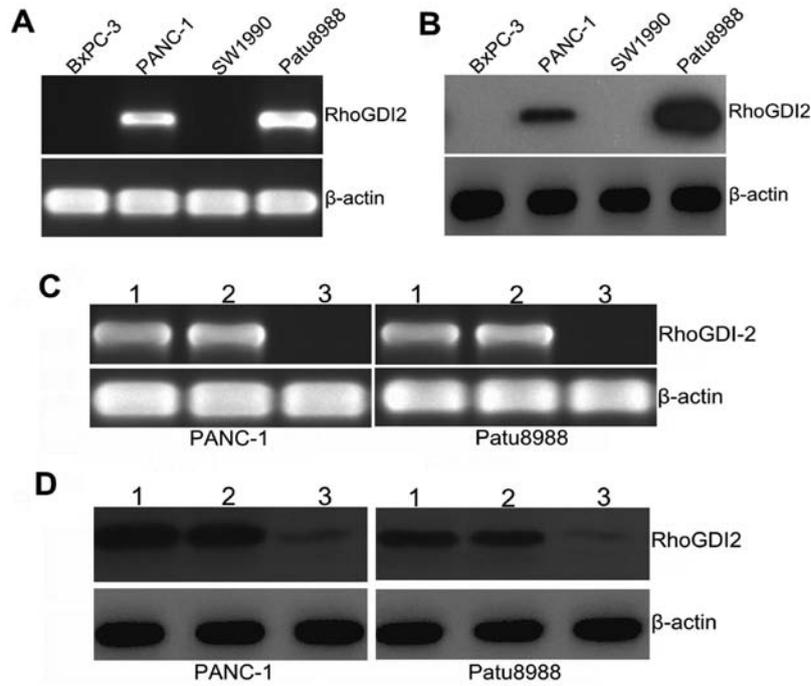


Figure 2. Silencing effect of Rho GDP dissociation inhibitor 2 (RhoGDI2). (A) Relative expression of RhoGDI2 mRNA in four PC cell lines (PANC-1, SW1990, Patu8988, BxPC-3) by quantitative PCR (qPCR). (B) Relative expression of RhoGDI2 protein in PC cell lines was assessed using western blotting. (C) The knockdown effect of RhoGDI2 mRNA was measured by qPCR. (D) The knockdown effect of RhoGDI2 protein was measured by western blotting. Lanes: 1, control; 2, RNAi-NC; and 3, RNAi-RhoGDI2.

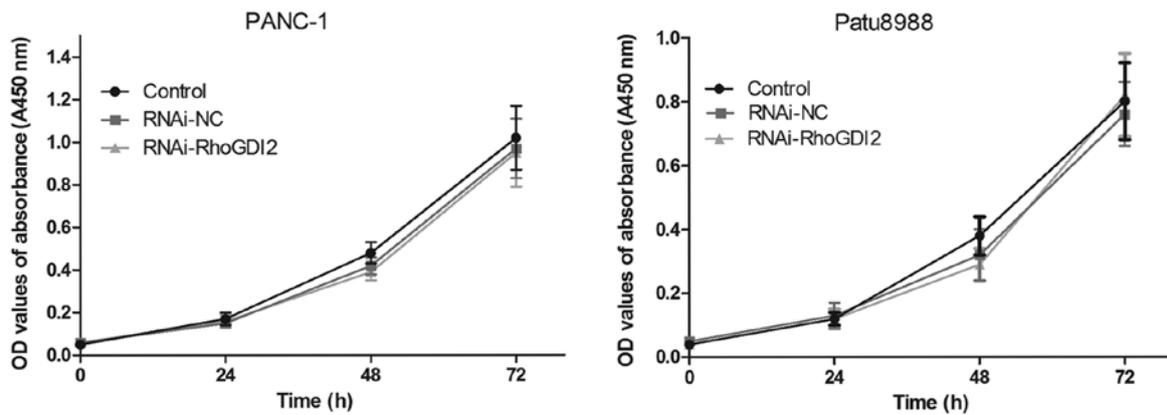


Figure 3. Cell proliferation following transfection with RNAi-RhoGDI2 or RNAi-negative control (NC) as analyzed by CCK8 assay. Data are the means \pm SD of five experiments.

expression in PANC-1 and Patu8988 cells using the RNAi method. As shown in Fig. 2C, the expression of RhoGDI2 mRNA was markedly decreased in the RNAi-RhoGDI2 group compared with the RNAi-NC group in PANC-1 and Patu8988 cells (both $P < 0.01$). A similar decrease was found at the protein level by western blotting (Fig. 2D), and the mean inhibition rate (the RNAi-RhoGDI2 group vs. the RNAi-NC group) was 78.3% in PANC-1 and 82.4% in Patu8988 (both $P < 0.05$). These findings indicated that downregulation of the RhoGDI2 gene was specific and efficient.

Silencing RhoGDI2 inhibits invasion in PC cells. Using the transfected cells, CCK8 assay was performed to examine

the effect of RhoGDI2 on PC cell proliferation. As shown in Fig. 3, there was no statistical significance in cell proliferation between the RNAi-RhoGDI2 and RNAi-NC groups ($P > 0.05$).

The effect of RhoGDI2 on cell motility and invasion was assessed using wound scrape and Transwell assays. The wound scrape assay was used to evaluate the effect of RhoGDI2 depletion on cell motility in PANC-1 and Patu8988 cells. Time course analysis of the wound closure showed that the re-established period of the monolayer was significantly shorter in the RNAi-NC group than that in the RNAi-RhoGDI2 group (both $P < 0.05$) (Fig. 4). Invasion assays were performed to determine whether RhoGDI2 is required for PC cell invasion

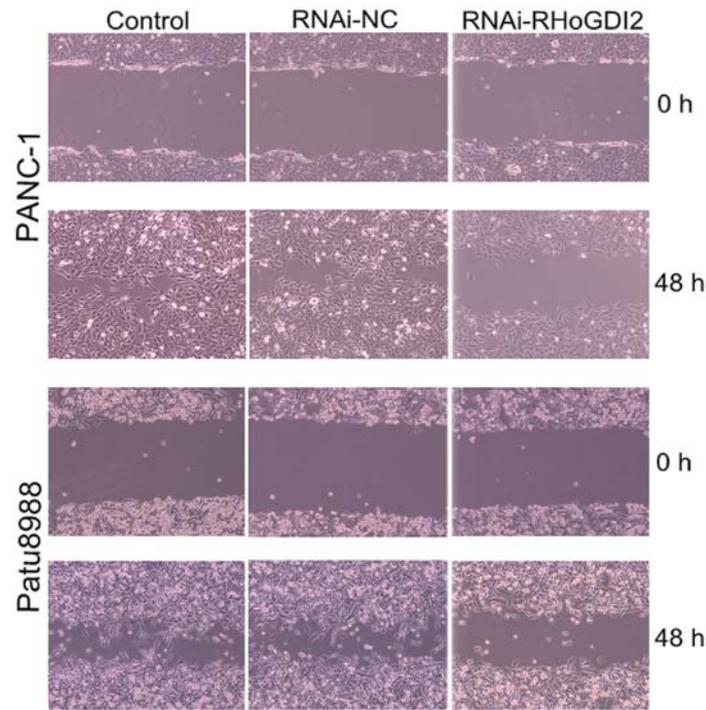


Figure 4. Cell migration as detected by the wound scrape assay. Monolayer images of cell migration in the wound scrape model at 0 and 48h are shown; original magnification, x100.

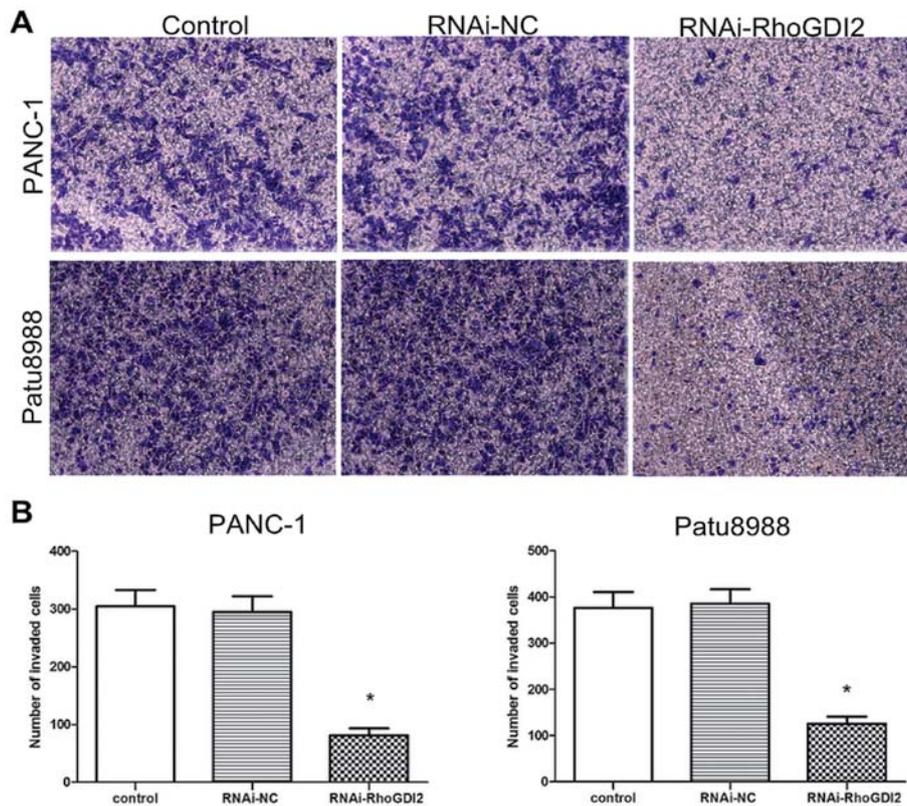


Figure 5. Cell invasion as detected by the Transwell assay. (A) Representative images of cell invasion captured under an inverted microscope (original magnification, x100). (B) The number of invaded cells in PANC-1 and Patu8988 cells. (*P<0.05).

based on the Boyden chamber assay. As shown in Fig. 5, depletion of RhoGDI2 significantly reduced cell invasion in the RNAi-RhoGDI2 group (81±12 cells per field for PANC-1 and

126±15 cells per field for Patu8988) compared to the RNAi-NC group (295±27 cells per field for PANC-1 and 386±31 cells per field for Patu8988) (both P<0.05).

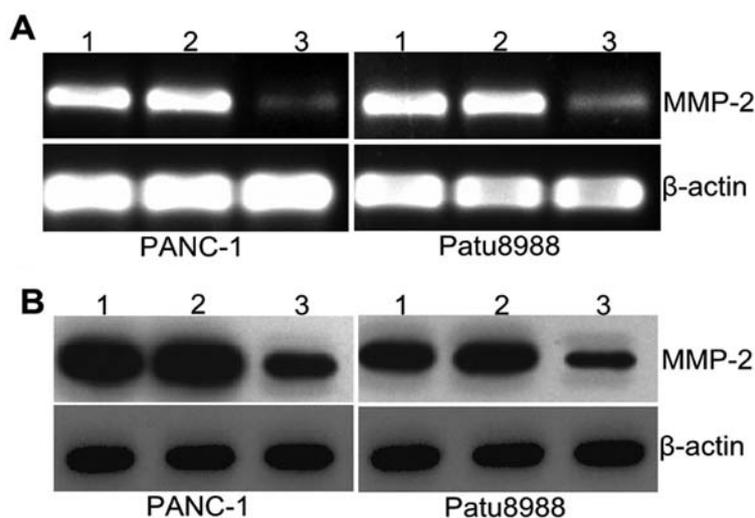


Figure 6. Silencing of Rho GDP dissociation inhibitor 2 (RhoGDI2) expression modulated the expression of matrix metalloproteinase 2 (MMP2) in pancreatic carcinoma (PC) cells as assessed by (A) qPCR and (B) western blotting. Lanes: 1, control; 2, RNAi-NC; and 3, RNAi-RhoGDI2.

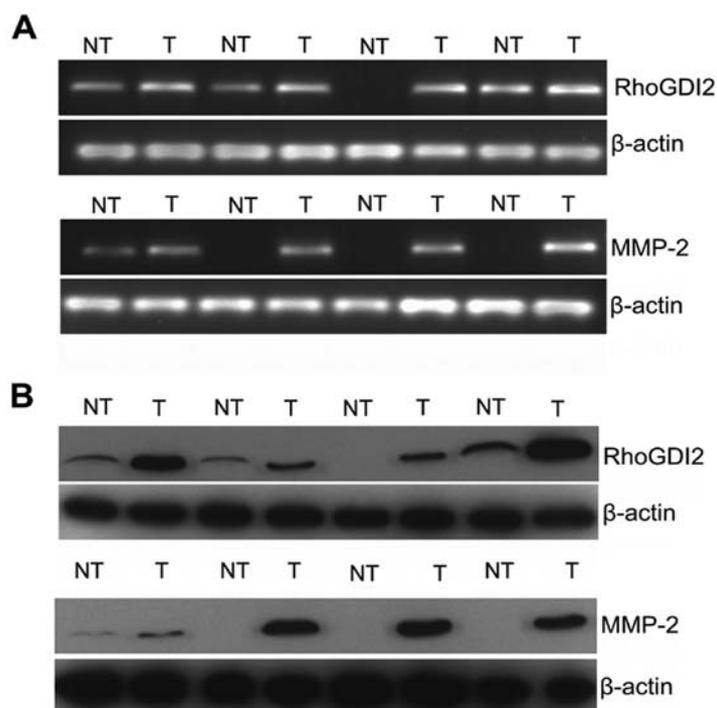


Figure 7. Correlation of expression of Rho GDP dissociation inhibitor 2 (RhoGDI2) and matrix metalloproteinase 2 (MMP2) in pancreatic carcinoma (PC). (A) Expression of RhoGDI2 and MMP2 mRNA was examined in PC and non-tumorous tissue (NT) by RT-PCR. (B) Expression of RhoGDI2 and MMP2 protein was examined in PC and NT by western blotting.

Silencing RhoGDI2 affects MMP2 expression. MMPs are involved in the degradation of extracellular matrix (ECM) and basement membranes. To determine whether RhoGDI2 promoted cancer cell invasion through MMPs, the expression of MMP2 was determined by qPCR and western blotting. Fig. 6A shows that MMP2 mRNA expression was significantly decreased in the RNAi-RhoGDI2 group compared with the RNAi-NC group in PANC-1 and Patu8988 cells (both $P < 0.05$). Results of western blotting revealed a similar decrease at the protein level (both $P < 0.05$) (Fig. 6A). The mean inhibition rate was 62.6% vs. the RNAi-NC group in PANC-1 and 60.7% in

Patu8988. These results indicated that RhoGDI2 contributed to the expression of MMP2 in PC cells.

RhoGDI2 is positively correlated with MMP2 in PC. To obtain clinical evidence of a positive correlation between RhoGDI2 and MMP2, the expression of 30 pairs of fresh PC and NT tissues were assessed by qPCR (Fig. 7A) and western blotting (Fig. 7B). The majority of PC exhibited overexpression of RhoGDI2 compared with NT at the mRNA and protein levels (both $P < 0.01$). Similarly, MMP2 was overexpressed in PC compared with NT at the mRNA and protein level (both

$P < 0.01$). Results of subsequent analysis revealed that RhoGDI2 expression was positively correlated with MMP2 expression at the mRNA level (Spearman analysis, $r = 0.627$, $P < 0.001$). Consistent results were obtained when we compared the data at the protein level (Spearman analysis, $r = 0.817$, $P < 0.001$). Our clinical study together with the experimental data confirmed that RhoGDI2 modulated the expression of MMP2.

Discussion

In this study, the expression of RhoGDI2 in four PC cell lines, 30 matched clinical fresh PC tissues and 60 pairs of clinical paraffin-embedded PC with clinical data was identified. We found a tendency towards the upregulation of RhoGDI2 in PC tissues compared to adjacent normal ones at the mRNA and protein levels. IHC results showed that the expression of RhoGDI2 was positively correlated with tumor size, clinical stage, lymph node metastasis and vascular invasion. In addition, depletion of RhoGDI2 in PANC-1 and Patu8988 cells by RNAi significantly inhibited cell motility and invasion *in vitro*, but had no effect on cell proliferation.

Three human RhoGDIs have been identified thus far: RhoGDI1, RhoGDI2 and RhoGDI3 (15-17). The proteins are key regulators of Rho GTPases function, such as cell motility, polarity and invasion. In contrast to RhoGDI1 and RhoGDI3, which are expressed ubiquitously, RhoGDI2 was reported originally to be expressed in hematopoietic cells, predominantly in B and T cells (18,19). However, accumulating evidence shows that RhoGDI2 is also differentially expressed in human cancers (20). In the majority of studies, RhoGDI2 has been shown to promote tumor cell invasion, angiogenesis and metastasis, such as in lung and gastric cancer (21,22). However, it can function as a metastasis suppressor gene in bladder cancer and Hodgkin's lymphoma (14,23). Our results showed that RhoGDI2 was overexpressed in PC and silencing RhoGDI2 inhibited cell invasiveness in PC cells. Those studies along with our findings indicate that the function of RhoGDI2 in cancer progression depends on different tumor types.

Although the reason for this discrepancy currently remains unclear, the conflicting role of RhoGDI2 may result from the dual roles of RhoGDI2 in the regulation of activities of Rho GTPases during cancer progression. RhoGDI2 bound the majority of Rho GTPases in the cytoplasm, maintaining Rho in an inactive form and inducing the disruption of Rho-dependent cell motility (24,25). By contrast, RhoGDI2 acted as an escort protein directing Rho GTPases to the membrane and associated with active forms of Rho, Rac and cdc42, maintaining Rho in an active form (26,27). Our results indicate that knockdown of RhoGDI2 expression in PC cells resulted in decreased cell motility and decreased invasion ability. However, the manner in which RhoGDI2 impacts on the activation of Rho GTPases requires further analysis. In addition to Rho GTPases activities, other mechanisms of RhoGDI2 in cancer have been reported in some studies. $\beta 1$ -integrin and cyclooxygenase-2, which play key roles in breast cancer progression, were identified as target genes of RhoGDI2 in breast cancer cells (12,28). PLC γ were validated to be required for RhoGDI2-mediated cell invasion and metastasis in gastric cancer cells (29).

Tumor invasion and metastasis are the critical characteristics in determining the aggressive phenotype of human

cancers, and require both tumor cell migration and degradation of matrix barriers (30). MMPs are involved in the degradation of ECM and basement membranes. Binker *et al* found that activation of Rac1 in PANC-1 cells were responsible for MMP2 secretion and activation (31). In the present study, we observed that the expression of MMP2 was significantly decreased following depletion of RhoGDI2 in PANC-1 and Patu8988 cells. Additionally, we found that MMP2 was positively correlated with RhoGDI2 expression in clinical PC tissues at the mRNA and protein levels. Thus, RhoGDI2 contributes to PC cell motility and invasion, at least in part by activating MMP2.

In summary, results of the present study demonstrated that the overexpression of RhoGDI2 was associated with PC progression. Depletion of RhoGDI2 in PC cells inhibited cell motility and invasion *in vitro*, at least in part by affecting the expression of MMP2. Our findings indicate that targeting RhoGDI2 by a genetic approach may provide a new strategy for the treatment in PC.

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