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Potential involvement of IQGAP1 in proliferation and metastasis of human pancreatic cancer

Yubiao Jin[1], Xiuhong Lv[1], Jilan Zhou[2], Jiawei Chen[1]

[1]Pathology Center, Shanghai First People's Hospital/Faculty of Basic Medicine, Shanghai Jiao Tong University School of Medicine, Number 100, Hai-Ning Road, 200080, Shanghai, China, [2]Department of Emergency, Yangpu Hospital, Tongji University School of Medicine, Shanghai, 200090, China

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1. ABSTRACT

IQGAP1, as a scaffold protein, integrates diverse cellular functions, including rearrangement of the actin cytoskeleton, cell adhesion, gene transcription and cell cycle regulation. IQGAP1 is elevated in a number of human cancer cells. However little is known about the expression of IQGAP in human pancreatic cancer and its association with cancer proliferation or metastasis. In the present study, we examined the expression of IQGAP1 in different pancreatic cancer cell lines and we found that IQGAP1 level is highly correlated with the degree of malignancy of pancreatic cancer cell metastasis. The proliferation, metastasis, motility and tumorigenesis in SW1990 human pancreatic cells were greatly impaired by down-regulating IQGAP1 expression with RNA interference. Mechanistic analysis indicated that Cdc42/Rac1 pathway might contribute to IQGAP1-mediated-pancreatic cell proliferation and tumorigenesis.

2. INTRODUCTION

The dynamic rearrangement of cell-cell adhesion is one of the major physiological events in organ development and cancer cell metastasis. Cell adhesion can be regulated by many different factors, for example cell scaffold proteins. IQ-domain GTPase-activating proteins (IQGAPs) are an evolutionally conserved scaffold protein family, involved in regulating various cellular processes ranging from cell adhesion, cell migration, extracellular signals and cell cycle (1). Among them, IQGAP1 is a multifunctional scaffold protein that binds to almost 100 other proteins. IQGAP1 is the more widely expressed protein compared to other family members (2, 3).

As a scaffold protein, IQGAP1 exerts its function through binding various partners to organize the components of a signaling pathway into complexes. IQGAP1 is an effector for Rac1 and Cdc42. These proteins act as molecular switchers to turn on (GTP-bound states) and off (GDP-bound states) signaling cascades (1). With its IQ domain, IQGAP1 can further interact with specific proteins such as actin (4), extracellular signal-regulated kinase 2 (ERK2) (5), mitogen-activated protein kinase (MEK) (6), calmodulin (7, 8), E-cadherin (9), β-catenin (10) and adenomatous polyposis coli (APC) (11), all of which are involved in cancer.

IQGAP1 is currently the only IQGAP gene reported as upregulated in cancer (3, 12). Numerous evidences have shown that IQGAP1 is highly associated with tumorigenesis. In some diffuse types of gastric cancer, IQGAP1 region is amplified in the tumor genomic DNA, resulting in a increase of IQGAP1 mRNA and protein level (13). In colon cancer, IQGAP1 was overexpressed in carcinoma tissues compared with normal counterparts and its expression was more abundant at the invasion fronts (14, 15). IQGAP1 expression is also increased in various other cancer cells, such as lung cancer, ovarian cancer and glioblastoma (16-18). Of note, higher expression of IQGAP1 appears to be related to higher spread ability. Strikingly, IQGAP1 expression is used as a biomarker for diagnosis of glioblastomas (17). Therefore, it's believed that IQGAP1 plays a very important role in tumorigenesis.

Pancreatic cancer is a very aggressive malignant tumor with a low survival rate, due to their early occurrence, high frequency of metastasis and relapse. The molecular mechanism underlying this high rate of metastasis is very unclear so far. Recently, Hage *et al* found that an association of IQGAP1 with β-catenin in pancreatic cancer (19). However, the expression and molecular mechanism of IQGAP1 in pancreatic cancer tumorigenesis remains illusive. In the present study, we examined the expression of IQGAP1 in different pancreatic cell lines. Through short-hairpin RNA (shRNA) knockdown, we assayed the effect of IQGAP1 on pancreatic cell proliferation and metastasis. We proposed here that IQGAP1 is essential for pancreatic cancer development and its function might be dependent on Rac1/Cdc42 activity.

3. MATERIALS AND METHODS

3.1. Cell cuture

The human pancreatic cell lines of SW1990, BxPC-3, PANC-1 and HPC-Y5 were cultured as previously described (20). Briefly, cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and were generally maintained at 37°C in a humidified atmosphere of 5% CO2 in air. The medium was replaced every 3 d.

3.2. Western blot

Protein expression for IQGAP1, β-actin, GAPDH, Rac-1 and Cdc42 was assayed by Western blot as described previously (19). Briefly, cells were washed by cold PBS for three times then whole cell extracts were prepared from cultured cells by homogenizing cells in lysis buffer [10 mM Tris-HCl (pH 7.5)], 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100) containing a cocktail of protease inhibitors. The supernatant was collected after centrifugation at 12000 g for 5 min, and quantitated by BCA method. 50 ug total protein was subjected to SDS-PAGE. Western blot analysis was performed using anti-IQGAP1 (Cell signaling technology, 1:2000), anti-β-actin (Sigmal-Aldrich), anti-GAPDH (Sigmal-Aldrich), anti-Rac1 (Santa Cruz Biotechnology, 1:1000) and anti-Cdc42 (Sigma-Aldrich, 1:2000) repsectively. Densitometry was performed using an image analyzer Fujifilm LAS4000 mini.

3.3. Cell proliferation assay

Cells were seeded at the density of 1000-10000/well into 96-well culture plates. Cell proliferation was determined at each time-point by adding 50 ul of 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltrazolium bromide) (MTT) into each well. Plates were incubated for an additional 4 h at 37°C and centrifuged before aspiration of supernatant and addition of 150 μl/well of DMSO. Absorbance at a wavelength of 490 nm in each well was measured using a Thermo Max microplate reader (Molecular Devices, Sunnyvale, CA).

3.4. Transwell migration and invasion assays

The migratory response of human pancreatic cells was determined by Transwell migration assay as described previously with slight modification (21). Briefly, assays were performed using a Transwell cell culture plate (Corning Costar, Rochester, NY, USA). The transwell inserts (8 μm pore size) were pre-coated with Matrigel (Becton Dickinson, Bedford, MA). The upper chamber contained cells in culture medium (5×10^5/ml) with 1% FBS, and the lower chamber contained culture medium with 20% FBS. Cells were incubated for 48 h. Nonmigrated cells were scraped from the upper surface of the membrane with a cotton swab, and migrated cells remaining on the bottom surface were counted after staining with 0.1% crystal violet.

3.5. Wound healing assay

Cells were cultured as confluent monolayers and wounded by scratching the confluent layer across the well with a sterile 200 μl pipette tip. Wounded monolayers were washed twice with PBS to remove detached cells and incubated for additional 24 h. Images of the wounds were taken and marked immediately at 0 h and 24 h, respectively. Wound healing was quantified using Image J software as the mean percentage of the remaining cell-free area compared with the area of the initial wound.

3.6. *In vivo* tumor formation

For subcutaneous tumorigenicity, 1×10^6 cancer cells were injected into the flanks of severe combined immunodeficient (SCID) mice. Tumor length (L) and width (W) were measured every 3 days, and tumor volume was calculated by $(W^2 \times L)/2$.

3.7. Rac1 activity assay

The amount of GTP-bound Rac1 was determined by using the Cdc42/Rac1 interactive binding region (CRIB)-domain of PAK1B (GST-PAK) as an activation-specific probe for activated Rac1 as described

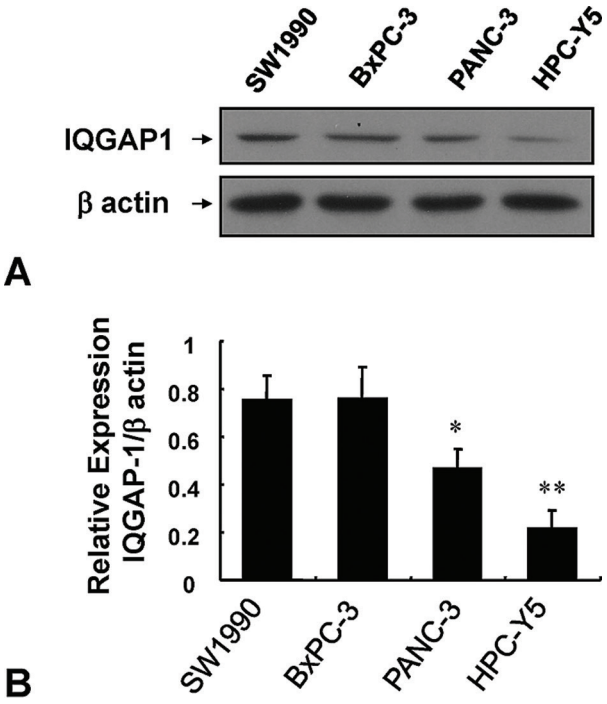

Figure 1. Expression of IQGAP1 in cultured pancreatic cancer cells. (A) Western blot detection of IQGAP1. Cell lysates from SW-1990, BxPC-3, PANC-3, and HPC-5Y cells were examined by Western blotting with an antibody to IQGAP1. Data are the mean of 3 determinations. (B) Expression of IQGAP1 relative to β-actin from A was quantitated.

in (22). Briefly, SW1990 cells were transfected with control or IQGAP1-shRNA and lysed. The lysates were then centrifuged at 20,000 g for 10 min at 4 °C and the supernatant was incubated with purified GST-CRIB beads for 1 h at 4 °C. The beads were washed and eluted with sample buffer. Immunoblotting with anti-Rac1 was then performed to detect the active Rac1.

3.8. Statistical analysis

The results were analyzed using SPSS 18.0 software (Chicago, IL, USA). Each experiment was repeated a minimum of three times. A two-tailed *t*-test was used to determine statistical significance. The results were presented as the means±S.D. *P*-values<0.05 were considered to be statistically significant.

4. RESULTS

4.1. IQGAP1 expression is correlated with the degree of tumor metastasis

IQGAP1 has been implicated to associate with many malignancies. In order to ascertain if the expression of IQGAP1 is upregulated in human pancreatic cancer, Western blot for IQGAP1 was performed in three cell lines which represented different malignant degrees of metastasis. Compared to that in normal pancreatic cells of HPC-5Y, IQGAP1 level was much higher in pancreatic cancer cells (Figure 1A). Interestingly, IQGAP1 is most abundant in SW1990 cells, which was 4-fold higher than HPC-5Y cells. BxPC-3 cells, like SW1900 cells, have approximately equivalent amounts of IQGAP1, while PANC-3 cells have much less IQGAP1 expression (Figure 1A and 1B). Considering SW1990 and BxPC-3 cells have the higher degree of metastasis, it is reasonable to propose that IQGAP1 level is positively correlated with cancer cell invasive potential.

4.2. IQGAP1 contributes to pancreatic cancer cell *in vitro* proliferation and invasiveness

To study the effect of IQGAP1 on pancreatic cancer tumorigenesis, we manipulated IQGAP1 expression in SW1990 cells. Lentivirus carrying control vector, IQGAP1 cDNA, or shRNA against IQGAP1 was introduced into SW1990 cells and stable cell lines were obtained. Compared with the vector cell lines, IQGAP1 overexpression cells displayed significantly increased IQGAP1 expression at the protein levels, whereas IQGAP1-shRNA cells showed dramatic decreased IQGAP1 expression. (Figure 2A)

Next, we explored the function of IQGAP1 expression on tumor cell growth. MTT assay was used to determine the proliferative ability. We found that enforced expression of IQGAP1 did not change the proliferation of SW1990 cells. Both control cells and IQGAP1 overexpression cells showed similar proliferation curve. In contrast, IQGAP1 knockdown significantly reduced the proliferation of SW1990 cells (Figure 2B). These data demonstrated that IQGAP1 is essential for pancreatic cancer cell proliferation.

To further examine the involvement of IQGAP1 in human pancreatic cancer cells migration, SW1990 cells with IQGAP1 overexpression or knockdown were subjected to Transwell assay. As showed in Figure 2C, IQGAP1 knockdown significantly deceased the cell migration ability as compared to the control cells. Consistently, IQGAP1 overexpression had no effect on SW1990 cells invasive capacity. Collectively, these data suggested that IQGAP1 contributes proliferation and invasion of pancreatic cancer cells.

To further validate the notion that IQGAP1 is essential for pancreatic cancer cell migration, we performed wound healing assay on SW1990 cells to assess the motility speed of cells. Wound closure was quantified from serial micrographs as shown in Figure 2D. After 24 hours, the average wound area relative to the original wound area was approximately 50% in control and IQGAP1 overexpressed cells, but was approximately 80% for IQGAP1 knockdown cells. In agreement with previous transwell assay, IQGAP1 knockdown impaired cell mobility by an average 80% decrease of migrated cell when compared with control SW1990 cells (Figure 2E). These results strongly indicate that IQGAP1 plays an

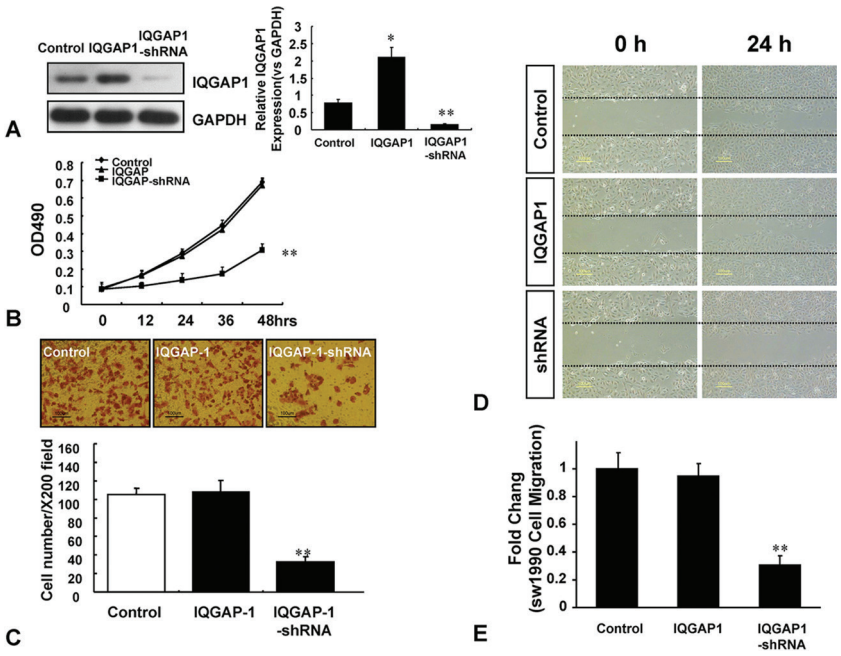

Figure 2. Effects of IQGAP1 on the proliferation and migration of SW1990 cells. (A) Stable IQGAP1-manipulated SW1990 cell lines were obtained. IQGAP1 expression was verified by Western blot. Relative IQGAP1 expression was also quantitated. (B) Cell proliferation after IQGAP1 overexpression and knockdown in SW1990 cells was measured using MTT assays. (C) Cell invasion ability was measured by Transwell assay and the number of migrated cells in three randomly chosen visual fields (magnification 20××) is given. Student t-test was performed to compare the difference. (D) The confluent monolayer of SW1990 cells was wounded and photographed at the indicated time points. (E) Relative SW1990 cells migration speed in wound-healing assay. **: P<0.01.

essential role in the migration and invasion ability of pancreatic cancer cells *in vitro*.

4.3. IQGAP1 contributes to *in vivo* pancreatic cancer cell proliferation

To assess the effect of IQGAP1 *in vivo*, a tumor xenograft study (n=9 per group) was conducted. 1×10^6 IQGAP1 overexpressing cells or IQGAP1-shRNA cells were subcutaneously injected into the flanks of nude mice. Similarly, tumor formation by IQGAP1 overexpressed cells (7/9 mice with obvious tumor) was comparable to that of control cells (6/9). However, mice receiving inoculation of IQGAP1-shRNA SW1990 cells showed no obvious subcutaneous tumorigenesis (3/9) (Figure 3A). Moreover, IQGAP1-shRNA cells had the least tumor numbers in nude mice, compared to control and IQGAP1 overexpressing cells (Data not shown). Tumor size from IQGAP1-shRNA cells was strikingly smaller. IQGAP1-shRNA injected cells also generated a significant decrease in tumor volume (Figure 3B). Collectively, these results suggested that knockdown of IQGAP1 inhibits pancreatic cancer cell xenograft formation and growth *in vivo*.

4.4. Knockdown of IQGAP1 alters the amounts of active Cdc42/Rac1 in pancreatic cancer cell

Activated Rac1 and Cdc42 positively regulate cadherin-mediated cell-cell adhesion and are essential for tumor cell migration and invasion (23). Given that IQGAP1 binds directly to Cdc42 and Rac1 *in vitro*, stabilizing the GTPases in their active form (19), we are asking whether Cdc42/Rac1 activity is affected in IQGAP1-knockdown cancer cells. As shown in Figure 4, stable overexpression of IQGAP1 in SW1990 cells does not increase the amounts of active Cdc42 and Rac1, whereas knockdown of IQGAP1 by shRNA led to a dramatic reduction of active Cdc42 and active Rac1 (Figure 4A and 4B). The total Rac1 and Cdc42 remained unchanged. These data indicated that IQGAP1, as a scaffold protein, facilitates and sustains GTP-bound Cdc42/Rac1.

4.5. Cdc42 and Rac1 participate in IQGAP1-mediated cell proliferation and metastasis in SW1990 cells

To further explore whether active Rac1 was functionally involved in IQGAP1-mediated pancreatic cancer cell proliferation and metastasis, constitutively active Rac1 (Rac1L61), a mutant that is defective in GTPase activity and exists constitutively in GTP-bound form in cells, was introduced into IQGAP1 knockdown SW1990 cells. In agreement with previous results, IQGAP1 knockdown resulted in markedly decrease of active Rac1. By contrast, Rac1L61 significantly restored active Rac1 level (Figure 5A) to the control level even in the presence of IQGAP1-shRNA. This indicates that

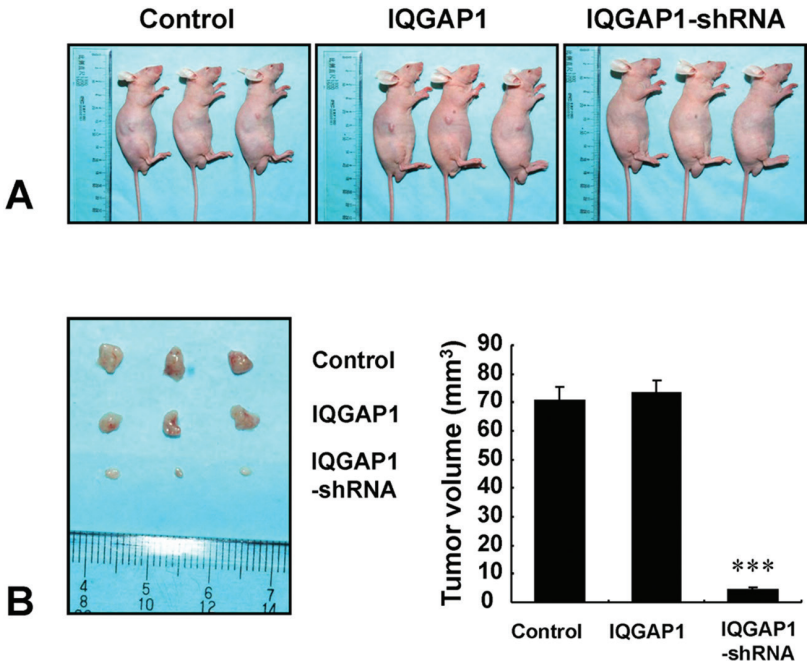

Figure 3. Knockdown of IQGAP1 expression reduces tumorigenic growth of SW1990 cells *in vivo*. (A) Representative images showed that the tumor formation is much less obvious in IQGAP1 knockdown injected mice. (B) Representative images showed that the tumor size of IQGAP1 knockdown groups in SW1990 cells was markedly smaller after tumor cells inoculation than that of control group.

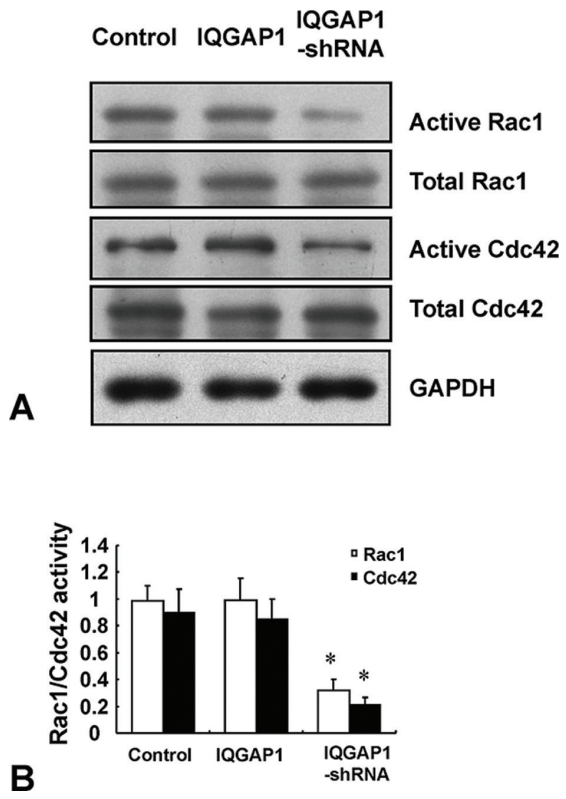

Figure 4. Active Rac1 and active Cdc42 were significantly deceased after IQGAP1 knockdown. (A) IQGAP1 or IQGAP1-shRNA stable SW1990 cells were assayed for active Cdc42 and Rac1. Total Rac1 and Cdc42 protein are shown as a loading control. (B) Bar graph represents averaged relative active Rac1 and active Cdc42 level. *: P<0.05.

constitutively active form of Rac1 successfully enhanced active Rac1 level after IQGAP1 knockdown, thus Rac1 probably acts as the downstream of IQGAP1.

Next, we examined the effect of enhanced active Rac1 on IQGAP1-mediated pancreatic cancer cell proliferation and migration. MTT assay showed that IQGAP1-shRNA significantly downregulated the cell proliferation, whereas, overexpression of Rac1L61 improved the pancreatic cancer cell growth, suggesting that active Rac1 recovered the inhibition effect of IQGAP1-shRNA. (Figure 5B)

To determine whether active Rac1 was also involved IQGAP1-mediated pancreatic cancer cell migration, we performed wound-healing assay under the condition that Rac1L61 was introduced in the stable IQGAP1-shRNA cells. Again, IQGAP1 knockdown reduced the number of migrated cells. As expected, constitutively active Rac1 enhanced the migration ability of SW1990 cells. Importantly, transfection of Rac1L61 in IQGA1-shRNA cells restored the cell migration ability. (Figure 5C and D)

Taken together, these data suggest that Rac1 activity contribute to the mechanism by which knockdown of IQGAP1 inhibits the tumorigenesis of pancreatic cancer cells.

5. DISCUSSION

IQGAP1 is a scaffold protein with implications in many diseases. Accumulating evidences have

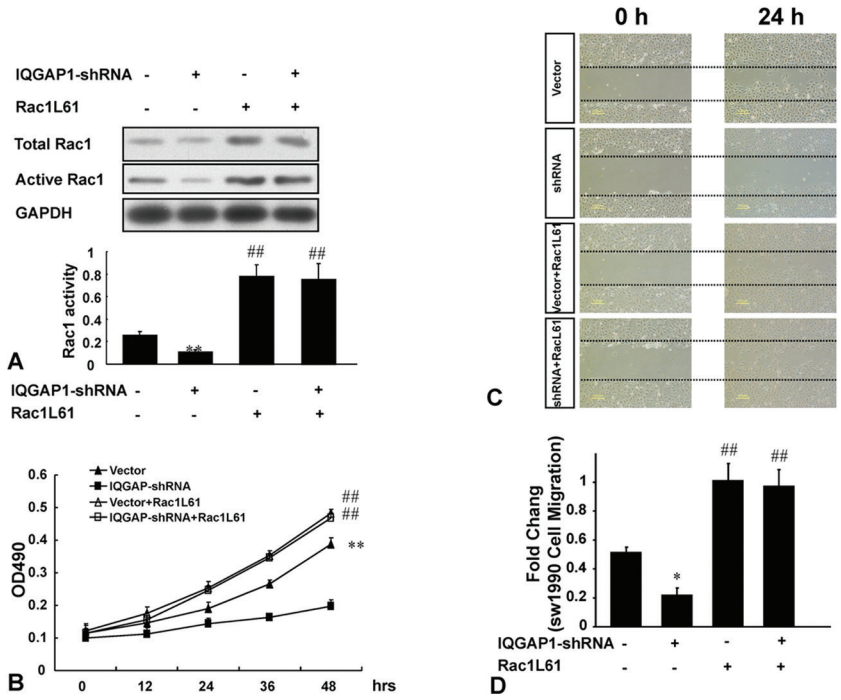

Figure 5. Active Rac1 is involved in IQGAP1-mediated tumorigenesis. (A) Active Rac1 were determined after transfection of constitutively active form of Rac1 in the IQGAP1-shRNA cells. Total Rac1 and GAPDH were used the loading control. (B) SW1990 cell proliferation was examined by MTT assay after Rac1L61 transfection in the IQGAP1-shRNA cells or control cells. (C) Images of scratch-wounded control and IQGAO1-shRNA SW1990 cells with or without Rac161L overexpression 0 h (immediately) or 24 h after scratching. (D) Graphic representation of the analysis of scratch-wound ability of SW1990 cells in C. *: P<0.05 compared with vector control. ##: P<0.01 compared with vector control.

demonstrated that IQGAP1 promotes cell proliferation, reduces cell-cell adhesion and increases cell migration, suggesting a predominant role in tumorigenesis of various cancer cells (12) (24). An enhanced expression of IQGAP1 has been documented for pancreatic carcinoma cell lines (25), but the molecular function of IQGAP1 in pancreatic cancer proliferation and metastasis is largely unknown. In the present study, we have analyzed the effect of IQGAP1 in pancreatic cancer cell proliferation and metastasis. Using shRNA to inhibit IQGAP1 expression in SW1990 cell, we revealed that IQGAP1 is responsible for human pancreatic cancer cell proliferation and metastasis *in vitro* and *in vivo*. Furthermore, we showed that enhancement of Rac1 activity by overexpress a constitutive active form of Rac1 can restore IQGAP1-mediated cancer cell development, suggesting that IQGAP1 and rac1/Cdc42 probably form a complex in pancreatic cancer signaling transduction.

The IQGAP family comprises three proteins in humans. IQGAP1 is the mostly studied member and the only reportedly unregulated in cancer cells. IQGAP2 and IQGAP3 harbor all the domains identified in IQGAP1, but their biological roles are poorly characterized. They may differ from IQGAP1 in tissue distribution, subcellular localization and interaction partners. IQGAP2 and IQGAP3, particularly IQGAP2, also appear to participate in neoplasia (24). In hepatocellular carcinoma, IQGAP2 was identified as a tumor suppressor linked to Wnt/b-catenin signaling pathway. IQGAP1 and IQGAP2 play opposing roles in hepatic carcinogenesis (26). In a population-based study on pancreatic cancer patients, Zeng *et al* found that IQGAP2 interacted with radiotherapy, indicating a casual relationship between IQGAP2 and pancreatic cancer prognosis (27)However, whether IQGAP1 and IQGAP2 retain functionally divergent roles in pancreatic cancer proliferation and metastasis remains to be further investigated.

Epithelial cells are characterized as strong cell-cell adhesion. The initiation of tumor metastasis requires lose of cell-cell adhesion and gain of invasion, which is enabled by epithelial-mesenchymal transition (EMT). The active, GTP-bound form of Rac1 regulates the formation of actin cytoskeleton structures, contributing to the formation of cell-cell adhesion (19, 23). In our present study, we found active Rac1 is involved in IQGAP1-mediated cancer cell proliferation and metastasis. Knockdown of IQGAP1 apparently decreased active Rac1 level. We did not look into the mechanism of IQGAP1 and Rac1 interaction in the pancreatic cancer tumorigenesis. However, since endogenous Rac1 can modulate E-cadherin complex and its function (19), it's believable that IQGAP1, as a scaffold protein contributes to pancreatic cancer cell metastasis by regulating cell-cell adhesion and cytoskeleton rearrangement.

Recently, IQGAP1, which brings together many members of the MAPK pathway, was found required in RAS-MAP kinase–driven tumors. Disrupting the function of IQGAP1 has been proven to be an effective way to target MAPK pathway with less side effects (28). Therefore, a better understanding of the molecular mechanisms of IQGAP1 in pancreatic cancer will aid in the development of new approaches to the prevention, and treatment of this deadly disease.

Interestingly, although IQGAP1 is overexpressed in pancreatic cancer cells, further enforced expressed of IQGAP1 does not increase the cell proliferation and invasiveness. This leads to an important speculation that the high level of IQGAP1 in pancreatic cancer cells is a late event rather than an early event. In other words, IQGAP1 is not likely the cause of tumor metastasis, but it is essential for tumor development as we showed in shRNA knockdown experiments. Moreover, IQGAP1 may exert different functions depending on the presence of binding partners and on the nature of different cells. In this regard, what triggers IQGAP1 upregulation in cancer cell progression and what is the key binding partner(s) that augment the tumorigenic role of IQGAP1 would be very fascinating questions.

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Abbreviations: IQGAPs, IQ-domain GTPase-activating proteins; ERK2, extracellular signal-regulated kinase 2; MEK, mitogen-activated protein kinase; APC, adenomatous polyposis coli; shRNA, short-hairpin RNA; EDTA, ethylenediaminetetraacetic acid; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltrazolium bromide; SCID, severe combined immunodeficient

Key Words: Pancreatic Cancer, IQGAPs, ERK2, APC

Send correspondence to: Jiawei Chen, Department of Pathology, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, China, Tel: 86-013386259539, Fax: 86-21-37798667, E-mail: jiaweichen2000@yeah.net