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Ran GTPase protein promotes human pancreatic cancer proliferation by deregulating the expression of Survivin and cell cycle proteins

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ABSTRACT

Ran, a member of the Ras GTPase family, has important roles in nucleocytoplasmic transport. Herein, we detected Ran expression in pancreatic cancer and explored its potential role on tumour progression. Overexpressed Ran in pancreatic cancer tissues was found highly correlated with the histological grade. Downregulation of Ran led to significant suppression of cell proliferation, cell cycle arrest at the G1/S phase and induction of apoptosis. *In vivo* studies also validated that result. Further studies revealed that those effects were at least partly mediated by the downregulation of Cyclin A, Cyclin D1, Cyclin E, CDK2, CDK4, phospho-Rb and Survivin proteins and up regulation of cleaved Caspase-3.

1. Introduction

Pancreatic cancer is one of the deadliest solid malignancies and is the fourth leading cause of cancer death in the world [1]. Despite developments in the detection and management of pancreatic cancer, only approximately 4% of patients survive 5 years after diagnosis [2]. Surgical resection provides the only chance of a cure for pancreatic cancer; however, unfortunately, 80–85% of patients present with advanced, unresectable disease [2]. Although many genes and molecular pathways have been reported to be involved in pancreatic carcinogenesis, it is critical to discover specific early detection biomarkers and molecular targets to fight pancreatic cancer [3,4].

The small GTPase Ran is a Ras-related GTPase that acts in diverse cellular processes. One of the functions of Ran is to regulate the nucleocytoplasmic transport of molecules through the nuclear pore complex [5–8]. In addition, Ran is involved in cell cycle progression through the regulation of the mitotic spindle assembly and cell cycle-related proteins [5,9–11]. It has been reported that a significant number of oncogenes, tumour suppressors and spindle assembly factors are regulated by Ran, some of which are closely related to tumourigenesis [7,12]. Ran is highly expressed in many types of malignancies including renal cell carcinoma, ovarian carcinoma and soft tissue sarcoma, whereas Ran presents at low levels in most normal tissues [13–15]. Recently, Xia et al. showed that acute silencing of Ran in tumour cells triggers defects in mitotic spindle assembly and induces cell apoptosis. However, Ran ablation in various normal cells is well tolerated and does not elicit mitotic defects or cell death [15]. These findings suggest that Ran might be an important protein involved in carcinogenesis. Survivin, a member of the inhibitors of apoptosis protein (IAP) family, which plays critical roles in the regulation of mitosis and apoptosis in cancer [16], has been considered a novel target of Ran [17].

To better understand the role of the Ran in pancreatic carcinogenesis, we first examined Ran expression in 62 cases of pancreatic cancer patients and found that Ran overexpression in cancer tissues correlated with tumour staging. Then, the effects of Ran on tumour growth rate, cell cycle and apoptosis were investigated through depletion of Ran expression in two aggressive human pancreatic cancer cell lines. We found that down-regulation of Ran could significantly suppress proliferation of pancreatic cancer cells *in vitro* and *in vivo* by regulating certain cell cycle effectors. Meanwhile, Ran inhibition also increased the cellular apoptosis rate in pancreatic cancer cells through reduced Survivin expression and enhanced Caspase-3 cleavage.

2. Materials and methods

2.1. Cell culture and tissue collection

The human pancreatic cancer cell lines PANC-1, AsPC-1, BxPC-3, PC-3, HUVECs were purchased from KeyGEN Co. (Nanjing, China). All of the cells were cultured in DMEM high glucose medium (Hy-

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clone, Beijing, China) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Hyclone, Logan, UT). Tissue specimens were collected from 62 patients with primary pancreatic cancer who underwent surgery in our hospital between 2009 and 2011. All cases of pancreatic cancer and adjacent non-tumour tissues were diagnosed clinically and pathologically.

2.2. Immunohistochemistry

We performed immunohistochemical staining with a polyclonal antibody against Ran (1:500; Santa Cruz Biotechnology, Delaware, CA) in pancreatic cancer tissues and matched adjacent non-cancerous tissues. As the negative control, the primary antibody was replaced with PBS. All sections were examined and scored independently by two experienced pathologists. Ran expression was evaluated according to the ratio of positive cells per specimen and staining intensity.

2.3. Construction of lentiviral vectors expressing Ran shRNA and cell infection

PANC-1, AsPC-1 and HUVECs cells were transduced with lentiviruses expressing Ran shRNA or scrambled non-target shRNA and then cultured in medium containing 3 μg/ml puromycin (Sigma, St Louis, MO) for 14 days to select for transduced cells. The lentiviral vectors were from GeneChemCo. (Shanghai, China). The sequence of human Ran shRNA was as follows: 5′-ACAGGAAAGUGAAGGCGAA-3′. Western blot and RT-PCR assays were performed to determine knockdown efficiency.

2.4. Western blot analyses

All proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were incubated with primary antibodies at 4 °C overnight. The antibodies included polyclonal antibodies against Ran (dilution 1:500; Santa Cruz Biotechnology, Delaware, CA) and monoclonal antibodies against β-actin (dilution 1:2000; Sigma, St Louis, MO), Cyclin A, Cyclin D1, Cyclin E, Cyclin-dependent kinase (CDK) 2, CDK4, p53 and p21 (diluted 1:500; Boster, Wuhan, China), Rb and phospho-Rb (diluted 1:500; Cell Signalling Technology, Beverly, MA), Survivin (dilution1:500; Bioworld, Nanjing, China), and cleaved Caspase-3 (dilution 1:200; Santa Cruz Biotechnology, Delaware, CA). Specific protein bands were visualised using an ECL system.

2.5. Real-time PCR

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, Carlsbad, California, USA) and dissolved in diethylpyrocarbonatetreated (DEPC) water. Ran expression levels were examined using the Takara Reverse TranscriptionSystem Kit (Takara Biotechnology Co. Ltd., Dalian, China) using Ran primers (sense: 5′-TACTGGAAAAACGACCTT-3′). Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as a loading control using the following primers (sense: 5′-GCACCGTCAAGGCTGAGAAC-3′). The PCR conditions were as follows: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min. All reactions were performed in triplicate.

2.6. MTT assay

The cells were plated in 96-well plates (Corning Costar Corp., Acton, MA, US) at 1×10^3 cells/well. Viable cells were detected at 1, 2, 3, 4, 5, 6 and 7 days after plating. The 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumromide (MTT) reagent was added into each well at 5 mg/ml and continuously incubated for 4 h. After adding 150 ml dimethylsulphoxide (DMSO) to each well, the absorbance was measured at 490 nm on a microplate reader BP800 (Biohit, Helsinki, Finland). Each experiment was performed in quadruplicate and was repeated 3 times.

2.7. Colony formation assay

Log phase cells were trypsinised into single cell suspension and transferred to 60-mm^2 plates (Corning Costar Corp., Acton, MA) at a density of 1×10^3 cells/well. The colonies were stained with Giemsa staining solution, and the total number of colonies was counted. Each assay was repeated 3 times.

2.8. Analysis of cell cycle and apoptosis using flow cytometry (FCM)

Infected cells and parental cells were harvested when they reached 70–80% confluency. The cells were washed with ice-cold PBS twice and then suspended in 1 ml of 70% alcohol at 4 °C. The DNA content of stained nuclei was analysed using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA). Cell cycle analyses were performed using Multicycle Software for cell cycle analyses. The cancer cells were induced by serum deprivation for 48 h and then trypsinised. Annexin V/FITC apoptosis detection Kit (Beyotime, Shanghai, China) was used to identify apoptotic and viable cells following the manufacturer's instructions. The percentage of apoptotic cells was calculated from the data originating from flow cytometry. The percentages of cell apoptosis = (Q2 + Q4)/100%.

2.9. Tumourigenicity assay

Female nude mice between 2 and 4 weeks of age were obtained from the Shanghai Institute for Biological Sciences (Shanghai, China). The PANC-1/shRNA cells andPANC-1/Ctr-shRNA cells were injected into the flanks of nude mice with 1×10^7 cells in 0.2 ml of PBS medium. The mice were then monitored for tumour volume every 5 days. Tumour volume was calculated according to the following formula: tumour volume = length × (width)^2/2. Each experimental group consisted of four mice.

2.10. Statistical analyses

All statistical analyses were conducted using SPSS version 17.0 software (SPSS, Chicago, IL)including the Mann–Whitney *U* test, Kruskal–Wallis *H* test, and Student's *t*-test. The minimal level of significance was defined as $P < 0.05$.

3. Results

3.1. Ran expression was significantly increased in pancreatic cancer

Ran expression was evaluated using immunohistochemistry in 62 primary pancreatic cancer tissues and matched, adjacent, noncancerous tissues. Ran was mainly located in the cytoplasm and nuclei of pancreatic cancer cells but only in the nuclear envelope of non-cancerous cells (Fig. 1A–F). As shown in Table 1, Ran staining was positive(+ to +++) in 62 (100%) pancreatic cancer tissue samples and 30 (48.4%) non-tumour tissue samples ($P < 0.01$).

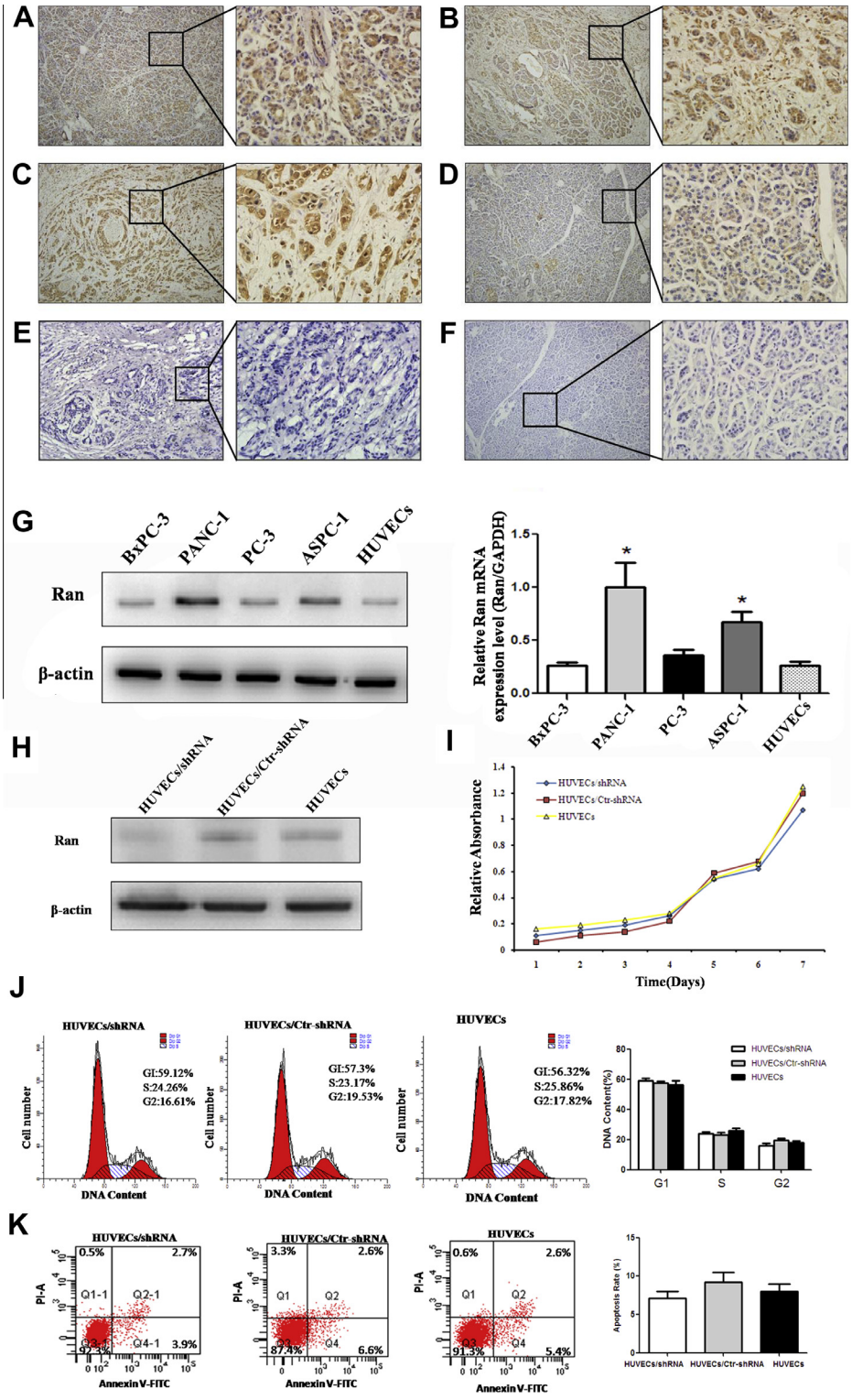

Fig. 1. Ran expression levels in pancreatic cancer tissues and cell lines. (A–C) Well differentiated, moderately differentiated, poorly differentiated pancreatic ductal cancer tissues exhibiting positive Ran immunostaining. (D) Normal pancreatic tissues exhibiting weak Ran immunostaining. (E, F) Negative controls of pancreatic cancer tissues and normal tissues using PBS instead of primary antibody. Representative immunohistochemical photographs were taken at different magnifications (100× & 400×). (G) Western blot and real-time PCR analysis of Ran in five cell lines, and the β-actin and GAPDH served as internal controls respectively. Pancreatic cancer cell lines PANC-1 and ASPC-1 compared with BxPC-3, PC-3 and HUVECs. (H) Ran expression levels were reduced in HUVECs/shRNA cells compared with the controls. (I–K) Examined cell proliferation, cell apoptosis, cell cycle of HUVECs/shRNA cells, and their control group cells by MTT assay and flow cytometry analysis respectively. Experiments were repeated in triplicate. $^{*}P < 0.05$.

As shown in Table 2, with respect to the histological stage, Ran expression in grade III and IV patients was substantially higher than in grade I and II patients ($P < 0.05$). Overall, Ran expression positively correlated with histological stage. However, there was no correlation between Ran expression and age, gender or tumour differentiation.

Table 1
Immunohistochemical analysis of Ran expression in pancreatic cancer and non-cancerous tissues.

Category	Total	Ran GTPase expression				Positive case	*P* value
		–	+	++	+++		
Pancreatic cancer tissues	62	0	13	20	29	62 (100%)	$P < 0.01^{**}$
Non-cancerous tissues	62	32	21	7	2	30 (48.4%)	

** $P < 0.01$ vs. non-cancerous tissue.

Table 2
Clinicopathological associations of Ran expression in pancreatic cancer.

Category	Total	Ran GTPase expressions			*P* value
		+	++	+++	
Gender					0.472
Male	38	7	12	19	
Female	24	6	8	10	
Age					0.842
⩽60 years	35	6	13	16	
>60 years	27	7	7	13	
Differentiation					0.325
Well differentiated	16	4	7	5	
Moderately differentiated	34	6	9	19	
Poorly differentiated	12	3	4	5	
TNM stage					0.039
I + II	35	9	14	12	
III + IV	27	4	6	17	

3.2. Ran promotes pancreatic cancer cell proliferation and inhibits cell apoptosis in vitro

We examined Ran expression in four pancreatic cancer cell lines (PANC-1, ASPC-1, PC-3 and BxPC-3) and human umbilical vein endothelial cells (HUVECs). Ran expression was higher in PANC-1, ASPC-1 cells which companied with k-Ras mutation [18], lower in PC-3 and BxPC-3 cells, and also lowly expressed in the normal cells HUVECs, as determined by Western blot and real-time PCR assays (Fig. 1G). We then used virus infection to explore the effect of Ran expression in pancreatic cancer cells and normal cells. PANC-1, ASPC-1, HUVECs cells were infected with lentiviral vectors expressing Ran shRNA. After cell infection and selection with 3 μg/ml puromycin for 2 weeks, Ran expression was analysed using Western blot assays and real-time PCR assays. The results showed that the protein levels and mRNA levels were substantially decreased in PANC-1 and ASPC-1 cells after Ran shRNA infection (Fig. 2A and B). And the expression of Ran in HUVECs cells also decreased (Fig. 1H). We designated the stably infected shRNA cells as PANC-1/shRNA cells, ASPC-1/shRNA cells, HUVECs/shRNA cells, and designated the infected scrambled non-target shRNA cells as PANC-1/Ctr-shRNA, ASPC-1/Ctr-shRNA cells, HUVECs/Ctr-shRNA. An MTT assay revealed that the proliferation rate of pancreatic cancer cells infected with shRNA was significantly lower than the control or parental cells (Fig. 2C, $P < 0.05$). The effect of Ran on pancreatic cancer cells colony-forming ability was analysed using colony formation assay. As indicated in Fig. 2D and E, the colony number of lower expression Ran cell lines is decreased almost twice compared to high expression Ran cell lines. This illustrates the Ran downregulation suppressed the colony-forming ability of both ASPC-1 and PANC-1 cells. Flow cytometry analysis showed that 59.22% of PANC-1/shRNA cells were in the G1 phase of the cell cycle compared with 41.20% of PANC-1/Ctr-shRNA cells and 40.28% of parental PANC-1cells (Fig. 2H, $P < 0.05$). In addition, 55.93% of ASPC-1/shRNA cells were in G1 compared with 40.53% of ASPC-1/Ctr-shRNA cells and 41.32% of parental ASPC-1 cells (Fig. 2I, $P < 0.05$). Representative images are shown in Fig. 2F and G. Therefore, downregulation of Ran expression in PANC-1/shRNA cells and ASPC-1/shRNA cells caused an increase in the number of cells in the G1 phase of the cell cycle, and the number of cells in the S phase was proportionally reduced. Apoptosis levels were also measured using FCM with Annexin V-FITC and PI staining. The cancer cells were induced through serum deprivation for 48 h and then trypsinised. The percentage of cells undergoing apoptosis was greatly increased in PANC-1/shRNA cells (20.1%) compared with PANC-1/Ctr-shRNA cells (7.30%) and parental PANC-1cells (5.40%; Fig. 3A, $P < 0.05$). The percentage of cells undergoing apoptosis was also increased in ASPC-1/shRNA cells (15.38%) compared with ASPC-1/Ctr-shRNA cells (7.52%) and parental ASPC-1 cells (6.80%; Fig. 3B, $P < 0.05$). Besides that, we also examined the proliferation, apoptosis, cell cycle of HUVECs/shRNA cells, HUVECs/Ctr-shRNA cells and parent cells by MTT assay and flow cytometry analysis respectively. The results showed that downregulation of Ran on normal cells dose not effect the cell proliferation, cell apoptosis and cell cycle (Fig. 1I–K).

3.3. Ran enhanced the growth and proliferation of pancreatic cancer cells in vivo

To investigate the effect of Ran on proliferative ability *in vivo*, a subcutaneous tumour formation assay was performed in nude mice. PANC-1/shRNA and PANC-1/Ctr-shRNA cells were injected subcutaneously into nude mice. After tumours appeared, they were measured every 5 days for 5 weeks. As shown in Fig. 3C, the tumours were significantly reduced in size in PANC-1/shRNA cells compared with PANC-1/Ctr-shRNA cells. A typical image of the cancer cell architecture of these tumours is shown in Fig. 3D. The growth curve for PANC-1/shRNA cells was significantly lower than the growth curve for PANC-1/Ctr-shRNA cells (Fig. 3E, $P < 0.05$ on days 25–35). Meanwhile, the average tumour weight in mice injected with the PANC-1/shRNAcells was dramatically lower than in mice injected with the control PANC-1/Ctr-shRNA cells (Fig. 3F). All these data demonstrated that Ran had the potential to promote the tumourigenicity of pancreatic cancer.

3.4. Inhibition of Ran expression induces cell cycle arrest and apoptosis in pancreatic cancer cells by deregulating cell cycle-related and apoptosis-related proteins

Flow cytometry analysis demonstrated that Ran depletion induced cell cycle arrest at the G1 to S transition as well as cell apoptosis. Then, we analysed the expression levels of cell cycle effectors and relevant apoptosis proteins through Western blot analysis in PANC-1/shRNA cells compared with the control and parental cells. As shown in Fig. 4, our results revealed that downregulation of Ran expression in PANC-1/shRNA cells was associated with a reduction in Cyclin A, Cyclin D1, Cyclin E, CDK2, CDK4 and phospho-Rb protein levels compared with the control and parental cells, whereas no changes were observed in total Rb, P53, or P21 in PANC-1/shRNA cells compared with control and parental cells. Furthermore, we confirmed that Ran inactivation induced the reduction of Survivin protein expression levels and Caspase-3 activation. Taken together, we concluded that the cell cycle G1 to S arrest induced by Ran inhibition in pancreatic cancer cells was at least

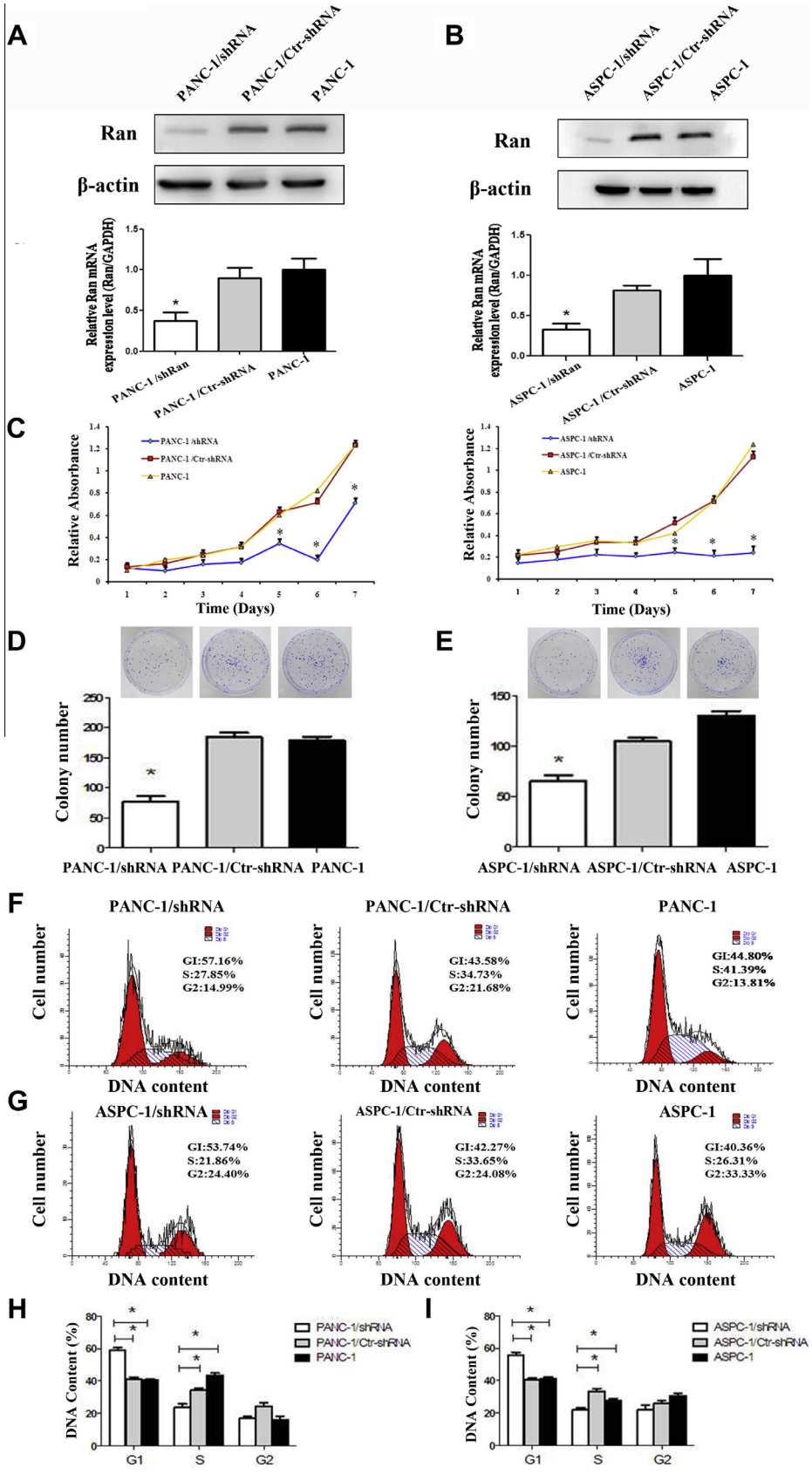

Fig. 2. Downregulation of Ran expression inhibited the pancreatic cancer cell growth rate and led to cell cycle arrest at the G1/S phase. (A, B) Ran expression levels were reduced, as determined through Western blot and real-time PCR assays in PANC-1/shRNA cells and ASPC-1/shRNA cells compared with the respective controls. β-Actin and GAPDH were used as internal controls. (C) The growth curves of PANC-1/ASPC-1 and its variants were determined using an MTT assay, and the growth curves were plotted. (D, E) The colony-forming ability of PANC-1/ASPC-1 cells and its variants was measured using a colony formation assay. (F, G) Cell cycle analysis of PANC-1/ASPC-1 cell and its variants was performed using flow cytometry. Representative images are shown in the figure. (H, I) The cell cycle data are shown in histograms. The values represent the mean (SEM) from at least three separate experiments. $^{*}P < 0.05$.

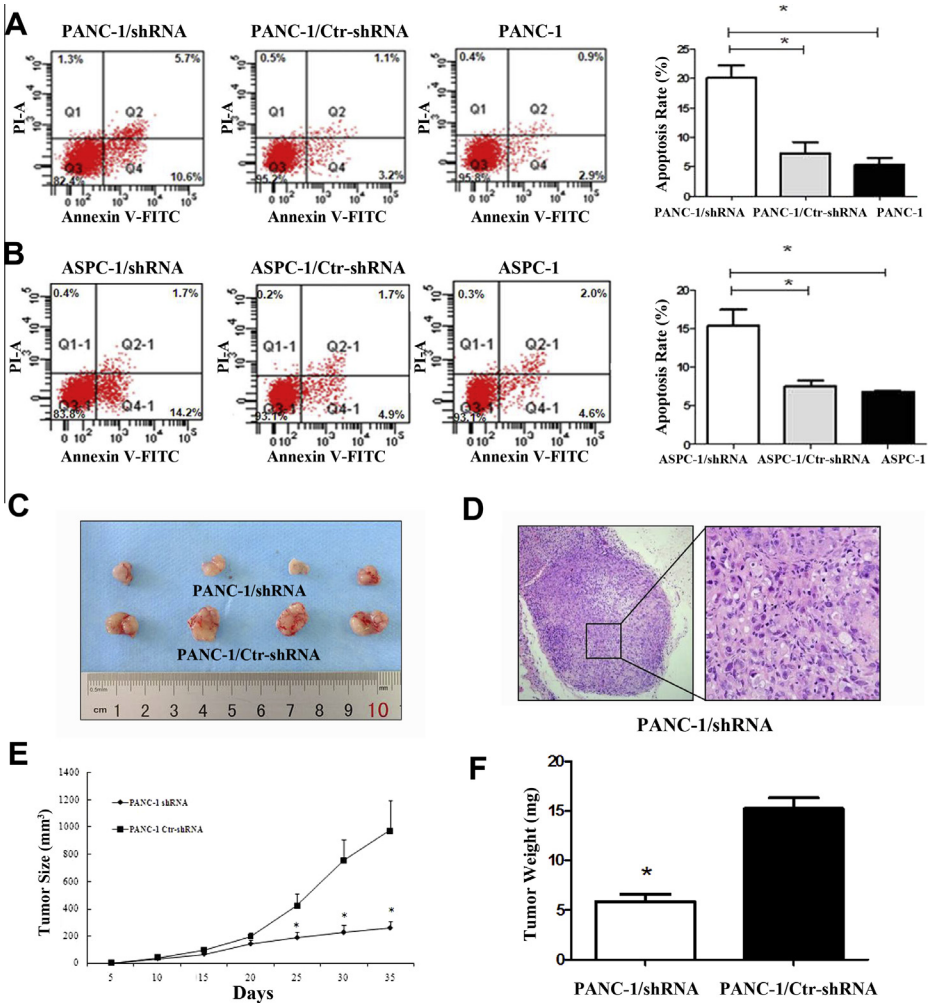

Fig. 3. Downregulation of Ran expression induced cellular apoptosis and inhibited the growth of pancreatic cancer cells *in vivo*. (A, B) PANC-1/ASPC-1 cells and its variants were harvested and subjected to cell apoptosis analysis using flow cytometry. Representative images are shown. The percentages of apoptosis are shown as histograms. (C) Representative images of tumours from mice injected with PANC-1 cells infected with shRNA and Ctr-shRNA are shown. (D) The HE staining of the nude mice tumours shows the typical cell architecture. (E) PANC-1cells infected with shRNA formed tumours with reduced volumes compared with Ctr-shRNA infected cells. Each data point is the mean value of four primary tumours. (F) Tumour weights were measured after 35 days of tumour inoculation. The values represent the mean (SEM) from at least three separate experiments. $^{*}P < 0.05$.

partly mediated by downregulation of Cyclin A, Cyclin D1, Cyclin E, CDK2, CDK4 and phospho-Rb proteins. Downregulation of Ran induced apoptosis in pancreatic cancer cells possibly through the reduction of Survivin protein expression and activation of Caspase-3.

4. Discussion

Although considerable advances have been made in surgical techniques and perioperative care, pancreatic cancer remains a clinical challenge because of the absence of effective methods for early diagnosis. Therefore, it is critical to discover specific early detection biomarkers and molecular targets to fight pancreatic cancer.

As previously noted, Ran GTPase, a small GTP-binding protein belonging to the Ras superfamily, was thought to play an important role in various types of human tumours. In this study, we first investigated the relationship between Ran and pancreatic carcinogenesis, and the experimental results suggested that Ran might play an important role in pancreatic carcinogenesis. We found that Ran expression was significantly higher in pancreatic cancer tissues and cells which companied with k-Ras mutation than in non-cancerous tissues and normal cells, and Ran expression positively correlated with the histological stage. Subsequent biological phenotypic and nude mice experiments demonstrated that inhibition of Ran significantly suppressed pancreatic cancer cell proliferation *in vitro* and tumourigenicity *in vivo* through cell cycle arrest at the G1 to S phase transition and increased cellular apoptosis. These results are consistent with the previous reports that depletion of Ran results in decreased tumourigenesis [13–15].

There is previous report that when components of the Ran system were perturbed, the genetic experiments in yeast indicated specific cell-cycle defects [19]. The study of the role for Ran in nucleocytoplasmic transport suggested that these defects might arise from disruption of the transport of crucial cell-cycle regulators or effectors [6,7], Ran also helps activate the major mitosis-promoting factor [20] and cyclin-dependent kinase-1 (CDK1)–Cyclin B protein kinase [9]. Moreover, Ran depletion could disrupt the formation of microtubule asters and spindles [5,10,21,22].

In this study, we demonstrated part of the underlying mechanisms by which Ran acts to induce cell cycle arrest and increase cellular apoptosis. There is substantial evidence that cyclins play important roles in regulating cell cycle progression [23,24]. The cyclin-dependent kinases (CDKs) are core components of the cell

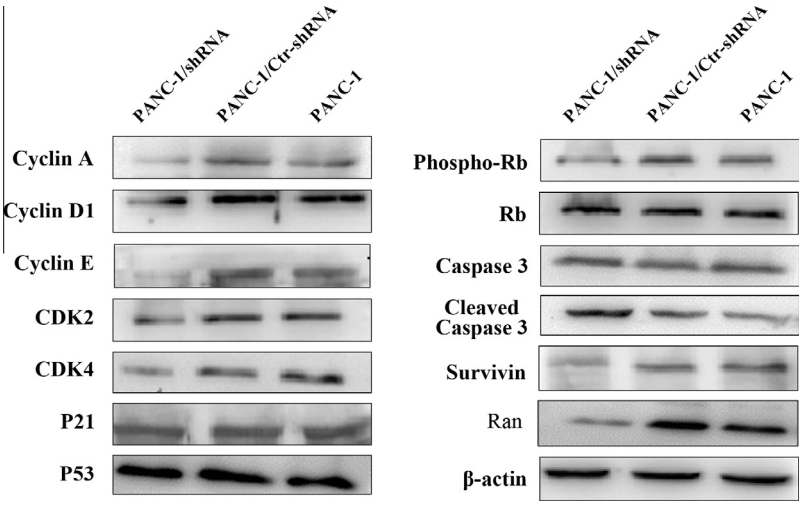

Fig. 4. The effects of Ran on cell cycle effectors and apoptosis-related proteins. The protein expression levels of Cyclin D1, Cyclin A, Cyclin E, CDK2, CDK4, phospho-Rb, Rb, p53, p21, Survivin, Caspase-3 and Ran were determined in parental and infected cells using Western blot analyses. β-Actin was used as an internal control. The data shown are representative of three independent experiments with similar results.

cycle machinery and are vital regulators driving cell cycle progression [25,26]. The Cyclin D1/CDK4 complex promotes Rb phosphorylation in the G1 to S phase transition [27,28], and Cyclin E/CDK2 phosphorylation of Rb in the late G1/early S phase promotes the G1 to S transition [29,30]. During S phase, the Cyclin A/CDK2 complex promotes S phase progression by maintaining Rb phosphorylation [31]. In our study, we also detected the expression changes of cell cycle-related and apoptosis-related proteins by Western blot analyses. Our results demonstrated that Ran downregulation could induce cell cycle arrest at the G1 to S phase transition and inhibit cell cycle progression by downregulating Cyclin D1, Cyclin E, Cyclin A, CDK4, CDK2, and phospho-Rb expression. However, the expression levels of P53 and P21 did not change in pancreatic cancer cells after knockdown of Ran. These results might be explained as different diseases or different cancers having different molecular signal pathways.

Survivin is a member of the inhibitor of apoptosis (IAP) gene family with dual roles in suppression of cell death [16,32,33]. Survivin is believed to protect cells from apoptosis by suppressing caspases directly or indirectly [34]. Survivin may also promote cell cycle progression by activating the CDK4/Cyclin D1 complex and enhancing RB phosphorylation to release the E2F transcription factor [35]. A recent study showed that acute silencing of Ran in various tumour cell types causes cell apoptosis, and this pathway is controlled by Survivin [15]. In this study, we found that downregulation of Ran in PANC-1/shRNA cells was associated with decreased Survivin expression. The result is consistent with Survivin as a potential Ran target gene [17]. Inhibition of Survivin reduced cell proliferation and induced apoptosis by down-regulating Cyclin D1 and phospho-Rb and activating Caspase-3. We also found that the expression of activated Caspase-3 was significantly increased in PANC-1/shRNA cells compared with the control and parental cells. Our results revealed that downregulation of Ran caused reduced the expression of Survivin, which can increase Caspase-3 cleavage to induce pancreatic cancer cell apoptosis. In addition to the above, Ran might also affect cell proliferation though regulation of the nucleocytoplasmic transport of pre-microRNA [36] and the transcription factor NF-κB [37]. Therefore, our further research direction will focus on the interactions between Ran and microRNA or transcription factors.

In conclusion, Ran is highly expressed in pancreatic cancer. Knockdown of Ran expression using shRNA inhibited pancreatic cancer cell proliferation and induced cell apoptosis by down-regulating Cyclin A, Cyclin D1, Cyclin E, CDK2, CDK4, phospho-Rb and Survivin protein expression and activating Caspase-3. Taken together, the current study provides Ran as a novel biomarker and target molecule for the diagnosis and treatment of pancreatic cancer.

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