



Silencing of the integrin-linked kinase gene suppresses the proliferation, migration and invasion of pancreatic cancer cells (Panc-1)

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Abstract

Integrin-linked kinase (ILK) is an ankyrin repeat-containing serine-threonine protein kinase that is involved in the regulation of integrin-mediated processes such as cancer cell proliferation, migration and invasion. In this study, we examined the effect of a lentivirus-mediated knockdown of ILK on the proliferation, migration and invasion of pancreatic cancer (Panc-1) cells. Immunohistochemical staining showed that ILK expression was enhanced in pancreatic cancer tissue. The silencing of ILK in human Panc-1 cells led to cell cycle arrest in the G0/G1 phase and delayed cell proliferation, in addition to down-regulating cell migration and invasion. The latter effects were mediated by up-regulating the expression of E-cadherin, a key protein in cell adhesion. These findings indicate that ILK may be a new diagnostic marker for pancreatic cancer and that silencing ILK could be a potentially useful therapeutic approach for treating pancreatic cancer.

Key words: E-cadherin, epithelial-mesenchymal transition (EMT), integrin-linked kinase (ILK), Panc-1 cell line, RNA interference (RNAi).

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Introduction

Pancreatic cancer is one of the most lethal cancers, with 5-year survival rates averaging less than 5% (Shaib *et al.*, 2006). Although several chemotherapeutic agents are active against pancreatic cancer, there is still no satisfactory chemical treatment for this disease (Yoganathan *et al.*, 2000; Chen *et al.*, 2010). Consequently, surgery is still the most effective treatment for pancreatic cancer. In most cases, however, surgery is not possible because the cancer cells have already invaded other tissues by the time of diagnosis. Less than 20% of the patients undergo surgical treatment, with the remainder receiving chemotherapy and/or radiotherapy, both of which are associated with serious side effects (Gunaratnam *et al.*, 2001). The development of new treatments with fewer side effects is therefore an important area of pancreatic cancer research.

Integrin-mediated cell adhesion regulates gene expression through the activation of transcription factors. These activations are mediated through integrin-linked kinase (ILK), an intracellular serine/threonine kinase that interacts with the cytoplasmic domains of integrin $\beta 1$ and $\beta 3$ subunits (Giancotti and Ruoslahti, 1999). Elevated ILK

expression has been observed in ovarian and lung cancer (Ahmed *et al.*, 2004; Hannigan *et al.*, 2005). ILK activity is stimulated by the binding of extracellular matrix components and growth factors. In addition, the association of ILK with tumor invasion is significantly correlated with all of the epithelial-mesenchymal transition (EMT) markers examined so far, including the loss of E-cadherin and Snail expression (Kang *et al.*, 2004; Barnes *et al.*, 2010). Consequently, the inhibition of ILK may represent a novel approach for treating pancreatic cancer. The aim of this study was to assess the expression of ILK in pancreatic cancer and examine the effects of siRNA-mediated knockdown of ILK in pancreatic cancer cells.

Material and Methods

Immunohistochemistry

Immunohistochemistry was done using formalin-fixed, paraffin-embedded tissue sections, as previously described (Ramos-Vara, 2005). Sixty-one pairs of resected pancreatic cancer tissue, 26 samples of corresponding adjacent tissue and four samples of normal tissue were fixed in 10% formalin solution and embedded in paraffin. Histological sections 3.5 μm thick were deparaffinized in xylene following dehydration with ethanol. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 20 min at room temperature (RT). After antigen retrieval, the sections

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were blocked with 5% bovine serum albumin for 20 min at RT and then probed with rabbit anti-ILK antibody (diluted 1:500; sc-20019, Santa Cruz Biotechnology Inc., CA, USA) at 4 °C overnight. After washing, the sections were incubated with biotinylated goat anti-rabbit immunoglobulins at RT for 1 h and immunoreactivity was detected using peroxidase-conjugated streptavidin and diaminobenzidine followed by counterstaining with Mayer's hematoxylin. The stained sections were examined and scored by a pathologist blinded to all clinical data. The tissues were scored positive when more than 10% of the cells reacted with the anti-ILK antibody and showed cytoplasmic staining.

Plasmids and reagents

A viral packaging system (pHelper 1.0 vector and pHelper 2.0 vector) and the vector pGCSIL/ILK-A were purchased from Genechem Co. Ltd (Shanghai, China). *AgeI*, *EcoRI* and SYBR Green Master Mix kits were purchased from TaKaRa (Dalian, China). An RNeasy Midi kit was obtained from Qiagen (Valencia, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Cambrex, MD, USA). Lipofectamine 2000, TRIzol and Super ScriptII reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA). The antibodies were obtained from Santa Cruz Biotechnology and included polyclonal anti-ILK (sc-20019, diluted 1:500), anti-E-cadherin (sc-33743, diluted 1:500) and anti-GAPDH (SC-32233, diluted 1:500).

Cell culture

Pancreatic cancer cells (Panc-1) and human embryonic kidney HEK293T cells were purchased from the American Type Culture Collection (ATCC). Both cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine and penicillin/streptomycin/amphotericin (100 µg each/mL) at 37 °C in a 5% CO₂ atmosphere.

Construction of ILK-specific shRNA lentivirus

The oligonucleotides encoding a negative control (NC) small interfering RNA (siRNA) (5'-TTCTCCGAACGTGTCACGT-3'), ILK siRNA (5'-CGAAGCTCAACGAGAATCA-3'), and stem-loop-stem oligos (short-hairpin RNAs, shRNAs) were synthesized, annealed and ligated into the vector *AgeI/EcoRI*-linearized pGCSIL-GFP. Expression of the lentiviral shRNA was confirmed by DNA sequencing. The plasmids generated were referred to as pGCSIL-shILK or -shNC. HEK293T cells (1×10^7) were seeded in 10 cm dishes and cultured for 24 h to 70%-80% confluence. Two hours before transfection, the medium was replaced with serum-free DMEM. Plasmids along with 20 µg of pGCSIL-shILK or -shNC, 15 µg of packaging vector pHelper 1.0 and 10 µg of

VSVG expression plasmid pHelper 2.0 were added to 200 µL of Opti-MEM and 15 µL of Lipofectamine 2000. Lentiviruses were harvested in serum-free medium two days after transfection, filtered and concentrated in primed Centricon Plus-20 filter devices (Millipore). Subsequently, Panc-1 cells were grown to 75% confluence and infected with ILK-shRNA lentivirus or control lentivirus at a MOI (Multiplicity of infection) of 30. The number of green fluorescent protein (GFP)-positive cells was determined microscopically four days post-transduction.

RT-PCR and real-time PCR analysis

RNA was extracted from infected cells using an RNeasy Midi kit according to the manufacturer's protocol. cDNA was synthesized using SuperScriptII reverse transcriptase. In brief, a mixture containing 1 µg of total RNA, 0.5 µg of oligo-dT primer (Shanghai Sangon) and nuclease-free water in a total volume of 15 µL was heated at 70 °C for 5 min and then cooled on ice for another 5 min. The mixture was supplemented with 2 µL of 10 buffer (supplied with the kit) and 200 U of SuperScriptII reverse transcriptase to a final volume of 20 µL followed by incubation at 42 °C for 60 min.

Real-time quantitative PCR was done using a SYBR Green Master Mix kit in a DNA Engine Opticon system (MJ Research, Waltham, MA, USA). Each PCR mixture containing 10 µL of 2 SYBR Green Master Mix, 1 µL of sense and antisense primers (5 µmol/µL) and 1 µL of cDNA (10 ng) in a total volume of 20 µL was run for 45 cycles that included denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. For relative quantification, $2^{-\Delta\Delta CT}$ was calculated and used as an indication of the relative expression levels. The primer sequences used to amplify the desired cDNA were: ILK forward and reverse primers: 5'-TCCACCTGCTCCTCA TCC-3' and 5'-CCTCATCAATCATTACACTACGG-3' and GAPDH forward and reverse primers: 5'-TGACTTCAACAGCGACACCCA-3' and 5'-CACCC TGTGCTGTAGCCAAA-3'. The PCR products were electrophoresed on 1.5% agarose gels, visualized by ethidium bromide staining and quantified using AlphaEase[®] gel image-analysis software (Alpha Innotech, San Leandro, CA, USA).

Western blotting

Western blotting was done using standard procedures (Towbin *et al.*, 1979). Cells were collected 96 h after infection and washed with ice-cold PBS. Whole-cell extracts were prepared using cell lysis buffer (20 mM Tris, pH 7.5, 0.1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 µg of aprotinin/mL and 10 µg of leupeptin/mL) and cleared by centrifugation at 12,000 g at 4 °C for 10 min. Equal amounts of protein were loaded onto 10% polyacrylamide gels for SDS-PAGE. After elec-

trophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). Following the blockade of non-specific sites, anti-ILK (1:500), anti-E-cadherin (1:500) and anti-GAPDH (1:500) antibodies were used to detect the respective proteins. Enhanced chemiluminescence detection was done in accordance with the manufacturer's instructions. Blots were visualized using an image analyzer and protein expression was quantified with an ImageQuant densitometric scanner (Molecular Dynamics).

Cell proliferation assay

Antiproliferative activity was assayed using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyl-tetrazolium bromide). For this, infected and non-infected Panc-1 cells were cultured in 96-well plates at an initial density of 2×10^4 cells/well. After 1, 2, 3, 4 and 5 days of infection, the cells were washed twice with phosphate buffered saline (PBS) and 100 μ L of MTT solution (5 mg/mL) was added to each well. After incubation for 4 h the MTT solution in each well was removed by suction and 100 μ L of dimethyl sulfoxide (DMSO) was added to solubilize the formazan salt. The optical density was measured at 490 nm with a UV microplate reader (Tecan Austria GmbH, Groedig, Austria). The relative cell viability was calculated by comparison with the NC cells.

Fluorescence-activated cell sorting

The cell cycle distribution was analyzed by fluorescence-activated cell sorting (FACS; Becton-Dickinson, Franklin Lakes, NJ, USA). The cells (1×10^6) were seeded onto six-well plates and allowed to attach overnight after which they were collected, washed in PBS and fixed in 70% cold ethanol. The cells were then treated with DNase-free RNase (100 μ g/mL) and incubated for 30 min at 37 °C. Propidium iodide (50 μ g/mL; Sigma) was added to the cell suspension and 10,000 fixed cells were analyzed by FACs.

Assay for apoptosis

Apoptosis was assessed using ApoScreen Annexin V Apoptosis kits (Southern Biotech Inc, Birmingham, AL). Cells infected with lentivirus carrying shILK and shRNA negative controls (NC) were grown to 75% confluence. The cells were then trypsinized, centrifuged, washed in 1x binding buffer and resuspended in 1 mL of Annexin V binding buffer. Subsequently, 5×10^5 cells (100 μ L of cell suspension) were stained with 5 μ L of Annexin V-APC followed by 5 μ L of PI. For each experiment, 20,000 cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Cell migration and invasion

Cell migration and invasion was assayed *in vitro* using a 24-well transwell unit (8 μ m pore size) containing

polyvinylpyrrolidone-free polycarbonate filters that were (invasion assay) or were not (migration assay) coated with 500 μ g/mL of BD Matrigel Basement Membrane Matrix (BD, Franklin Lakes, US). The cells were placed in the upper compartment of the migration chamber and allowed to attach for 8 h prior to incubation in FBS-free medium for 24 h at 37 °C in a 5% CO₂ atmosphere. The lower compartment of the migration chamber contained DMEM with 10% FBS. After incubation, the filter inserts were removed from the wells and the cells on the upper side of the filter were removed using cotton swabs. The cells that migrated to the lower surface of the membrane were fixed with methanol and stained with 0.5% crystal violet for 10 min. The phenotypes of the migrating cells were determined by counting the cells that migrated to the lower side of the filter using a Leica DM6000B microscope at a magnification of 100x (Leica Microsystems Wetzlar GmbH, Germany). Three migration chambers were used per condition. The number of migrating cells was calculated by averaging the number of cells in at least three bright fields for each of three filters.

Statistical analysis

The results are presented as the mean \pm SD of at least three independent experiments done on separate days using freshly prepared reagents. Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. A value of $p < 0.05$ indicated significance. The statistical software package, SPSS v.16 (SPSS Inc., Chicago, Illinois, USA), was used for the analysis.

Results

Table 1 and Figure 1 summarize the expression of ILK in pancreatic cancer tissue, adjacent tissue and normal pancreatic tissue based on immunohistochemical analysis. No ILK expression was detected in 7 (11.5%) of the 61 pancreatic cancer tissue samples whereas 21 samples (34.4%) were hadro-positive and 19 samples (31.1%) were positive; the remaining samples were weakly positive. In contrast to pancreatic tissue, 88.5% and 100% of the adjacent and normal tissue samples, respectively, showed no ILK expression. These findings indicate that ILK expression was enhanced in pancreatic cancer tissue compared to normal and adjacent pancreatic tissues. This enhanced ILK expression may be related to the pathogenesis and progression of pancreatic cancer.

To suppress ILK expression, Panc-1 cells were infected with a lentivirus expressing ILK-specific siRNA and GFP. After 96 h, more than 85% of cells expressed GFP, indicating successful infection (Figure 2A). RT-PCR showed that the ILK mRNA levels in ILK siRNA-infected cells (KD) were significantly lower than in cells infected with pGCSIL-shNC (NC) (Figure 2B). Infection with ILK

siRNA also markedly attenuated ILK protein expression (Figure 2C). These findings indicate that siRNA directed towards ILK was effective in specifically knocking down the ILK gene in Panc-1 cells.

Table 1 - Expression of ILK in pancreatic cancer tissue and normal tissue.

Tissue type	Number of cases	ILK expression			
		Negative (-)	Weakly positive (+)	Positive (++)	Hadro-positive (+++)
Cancer	61	↓ 7 (11.5%)	↑ 14 (23%)	↑ 19 (31.1%)	↑ 21 (34.4%)
Adjacent	26	20 (77%)	3 (11.5%)	3 (11.5%)	0 (0%)
Normal	4	4 (100%)			

↓, decreased ILK expression in cancer tissue compared to adjacent and normal tissues.

↑, enhanced ILK expression in cancer tissue compared to adjacent and normal tissues.

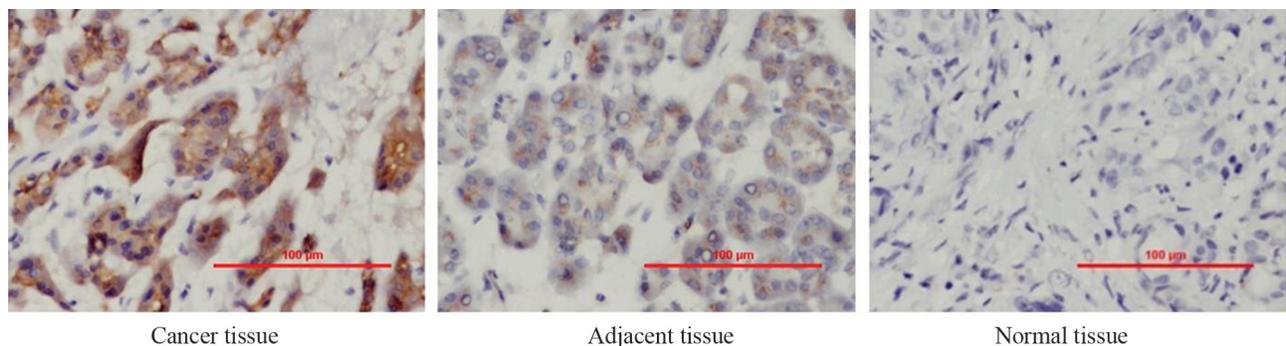


Figure 1 - Immunohistochemical analysis of ILK expression in prostate cancer tissue, adjacent tissue and normal tissue. Scale bars are in micrometers.

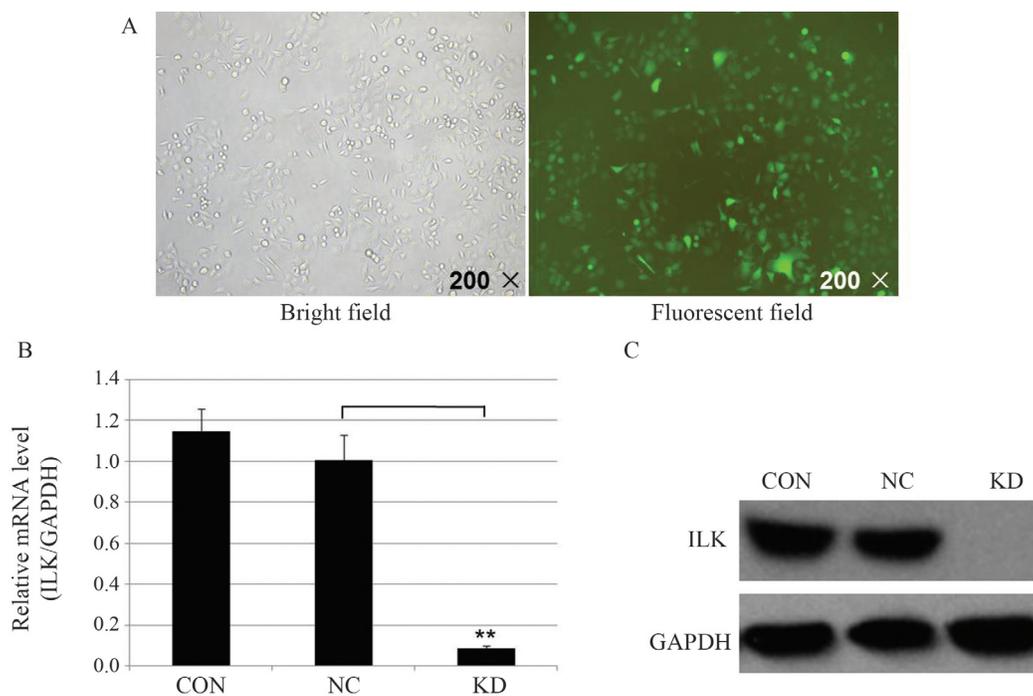


Figure 2 - ILK siRNA suppresses ILK mRNA and protein expression in Panc-1 cells. (A) GFP fluorescence (right panel) of Panc-1 cells 96 h after infection with lentivirus containing pGCSIL-shILK or -shNC (magnification: 200x). (B) Quantitative real-time PCR assessment of ILK mRNA levels after infection with pGCSIL-shILK compared to negative control (NC) and control (CON) cells. (C) Western blot for ILK protein expression levels in Panc-1 cells infected with ILK siRNA compared to NC and CON cells. The columns in (B) represent the mean ± SD of three independent experiments. **p < 0.01 compared to the control.

The MTT-based cell proliferation assay revealed markedly less proliferation in pGCSIL-shILK infected Panc-1 cells compared to pGCSIL-shNC infected cells at 3, 4 and 5 days post-infection (Figure 3A). The optical density corresponding to the number of cells was four-fold lower in shILK-infected cells compared to shNC-infected cells. These findings indicate that ILK is necessary for Panc-1 cell proliferation.

The deletion of ILK resulted in a significant accumulation of Panc-1 cells in the G₁ phase and a decrease in the number of cells in the S and G₂/m phases (Figure 3C). Significantly more pGCSIL-shILK-infected cells were in the G₁ phase compared to pGCSIL-shNC-infected cells (65.2% vs. 55.1%; $p < 0.05$). These results indicated that ILK knockdown caused cellular arrest in the G₁ phase of the cell cycle (Figure 3D). In Panc-1 cells infected with shILK the level of apoptosis reached 22.2%, as shown by FACS of annexin V-stained cells (Figure 3B). This finding suggested that ILK suppressed cell proliferation possibly by inducing apoptosis.

Cancer metastasis requires invasiveness by cancer cells. We used crystal purple staining and the transwell assay to examine the effect of ILK knockdown on the migration and invasion of Panc-1 cancer cells. As shown in Figure 4A,B, the migration of shILK-infected pancreatic cancer cells was significantly reduced ($p < 0.01$) compared to the negative control cells. The migration rate was approximately six-fold lower after ILK knockdown. Interestingly, the invasion rate of shILK-infected cells was also significantly lower ($p < 0.05$) than in shNC-infected cells.

E-cadherin plays an essential role in normal physiological processes and in pathological states such as epithelial-mesenchymal transition (EMT). Down-regulation of E-cadherin usually leads to tumor dedifferentiation, infiltration and the metastasis of cancer cells. We therefore used western blotting to examine the effect of shILK transfection on E-cadherin expression. Deletion of ILK led to enhanced E-cadherin expression (Figure 4C) and this may have contributed to the reduction in the rates of invasion and migration.

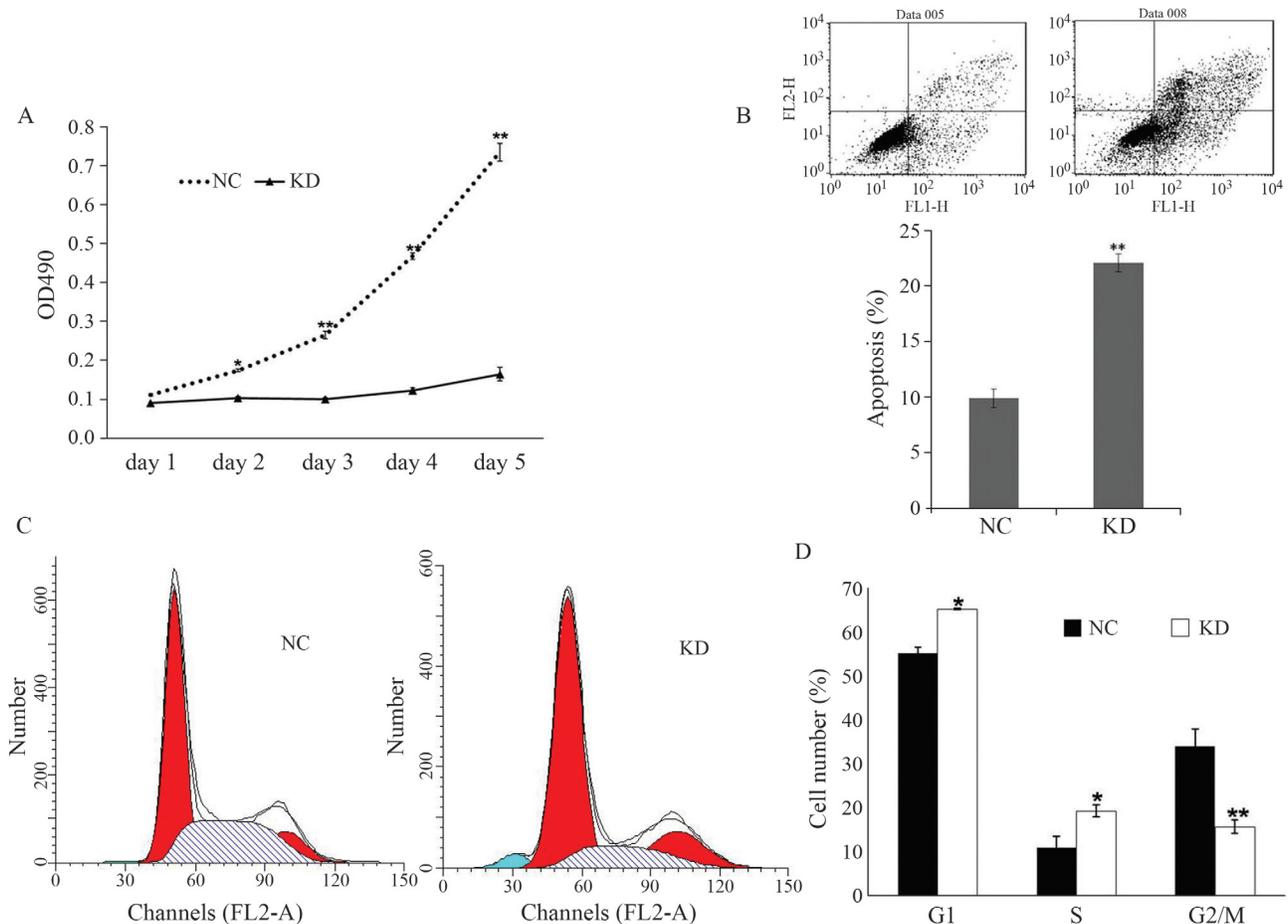


Figure 3 - ILK siRNA infection suppresses the proliferation of Panc-1 cells and induces cell cycle arrest in G₁ phase. (A) Cellular proliferation of ILK siRNA-infected (knockdown – KD) and negative control (NC) cells. (B) FACS analysis of Annexin V-APC stained KD or NC cells 96 h after transient infection. (C) Flow cytometric analysis of the cell cycle in NC and KD Panc-1 cells 96 h after transient infection. (D) The percentage of KD and NC Panc-1 cells in each phase of the cell cycle. The columns or points represent the mean \pm SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to NC cells.

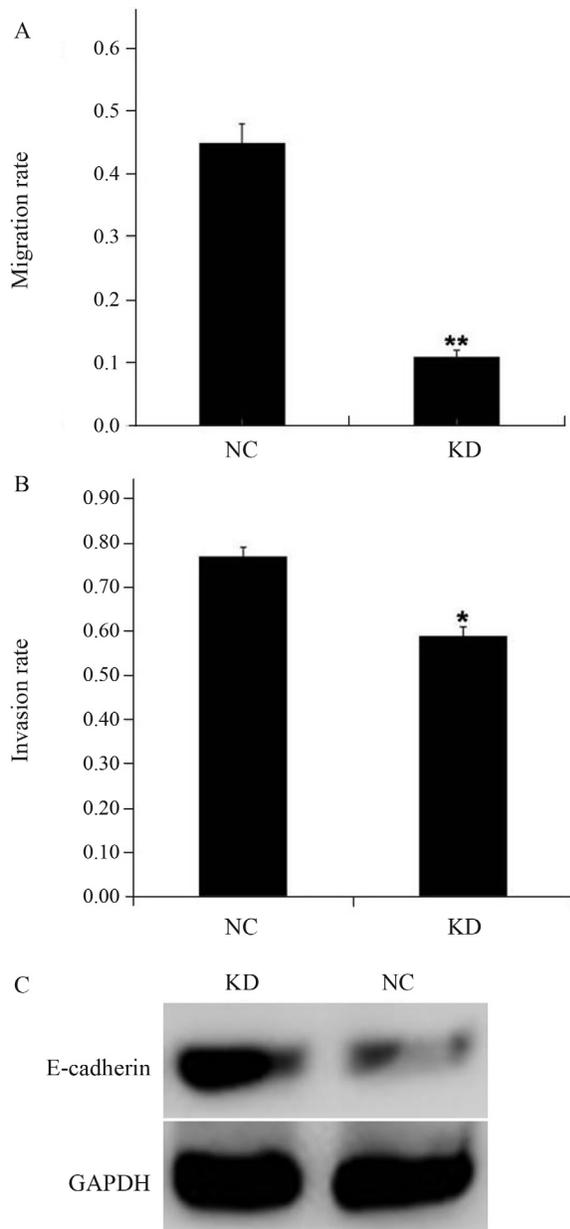


Figure 4 - ILK deletion induces the over expression of E-cadherin. (A) Migration distance and (B) invading cells number of shILK-infected (knockdown – KD) and shNC-infected (NC) Panc-1 cells. (C) Enhanced protein expression of E-cadherin in KD Panc-1 cells. The columns in (A) and (B) represent the mean \pm SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to NC cells.

Discussion

Pancreatic cancer is the fourth most common cause of cancer-related deaths worldwide (Hariharan *et al.*, 2008). Pancreatic cancer often has a poor prognosis, with the 1- and 5-year relative survival rates are 25% and 6%, respectively, for all stages combined. The mechanisms involved in the invasion and metastasis of pancreatic cancer are being intensively studied.

ILK is an intracellular protein that interacts with the cytoplasmic domains of the integrin $\beta 1$ and $\beta 3$ subunits. Previous work has established a central role for ILK in connecting integrins to the actin cytoskeleton (Brakebusch and Fassler, 2003). Increased levels of ILK expression have been observed in a variety of human tumors such as malignant melanoma, prostate cancer, colon cancer, thyroid cancer, lung cancer, Ewing's sarcoma and primitive ectodermal tumors (David and Parham, 2001). ILK expression is elevated in virtually all of these cases, indicating the ILK could be a useful diagnostic marker for these types of cancer.

The adhesion and metastasis of cancer cells in the extracellular matrix are also regulated by ILK. EMT has long been known to play a significant role in tumor progression, dedifferentiation, infiltration and metastasis. There is increasing evidence that ILK is a critical mediator of the induction of EMT. E-cadherin, a member of the cadherin superfamily of calcium-dependent, transmembrane glycoproteins, plays an essential role in normal physiological processes and in pathological states such as EMT. ILK may regulate E-cadherin expression indirectly by modulating the transcription of *snail* that in turn represses E-cadherin expression (Kang and Massague, 2004; Barnes *et al.*, 2010). The down-regulation of E-cadherin leads to reduced cell-cell adhesion. Under confluent conditions, these cells detach, grow in suspension and undergo EMT that is characterized by reduced expression of E-cadherin and increased expression of Snail. The over-expression of ILK increases the invasive potential of cancer cells by stimulating the expression of invasion-related genes such as MMP-9 (Matsui *et al.*, 2012). Based on studies *in vivo*, Yau *et al.* (2005) reported that ILK plays a prominent role in oncogenic phosphatidylinositol 3-kinase/PKB signaling and suggested that ILK inhibitors might be useful for treating pancreatic cancer patients. Knockdown of the ILK gene may inhibit IL-1 α -induced activation of the MAPK/AP-1 signaling pathway by regulating GSK-3 phosphorylation (Kumar *et al.*, 2004). This regulation of AP-1, a major transcription factor that regulates MMP-9 expressions, could have contributed to the lower invasiveness of ILK-repressed pancreatic cancer cells (Troussard *et al.*, 2000).

In conclusion, the results of this study show that ILK up-regulation plays a major role in the survival and proliferation of pancreatic cancer (Panc-1) cells and that silencing the ILK gene suppresses the proliferation, migration and invasion of these cells. The reduced migration and invasion of Panc-1 cells may be related to the enhanced expression of E-cadherin, a key protein involved in cell-cell adhesion. Together, these findings suggest that ILK gene suppression could be a potentially useful approach for treating pancreatic cancer.

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