RESEARCH ARTICLE

Knockdown of Ezrin by RNA Interference Reverses Malignant Behavior of Human Pancreatic Cancer Cells in Vitro

Zhi-Qiang Zhong[1], Mao-Min Song[1]*, Ying He[2], Shi Cheng[1], Hui-Sheng Yuan[1]

Abstract

Background: Pancreatic cancer is one of the most aggressive tumors with a dismal prognosis. The membrane cytoskeletal crosslinker Ezrin participates in several functions including cell proliferation, adhesion, motility and survival. There is increasing evidence that Ezrin is overexpressed in vast majority of malignant tumors and regulates tumor progression. However, its roles in pancreatic cancer remain elusive. Methods: Three pairs of specific Ezrin siRNAs were designed and synthetized and screened to determine the most efficient one for construction of a hairpin RNA plasmid targeting Ezrin. After transfection into the Panc-1 pancreatic cancer cell line, real-time quantitative PCR and Western blotting were performed to examine the expression of mRNA and protein. The MTT method was applied to examine the proliferation and the drug sensibility to Gemcitabine. Flow cytometry was used to assess the cycle and apoptosis, while capacity for invasion was determined with transwell chambers. Furthermore, we detected phosphorylated-Erk1/2 protein and phosphorylated-Akt protein by Western blotting. Results: Real-time quantitative PCR and Western blotting revealed that Ezrin expression was notably down-regulated at both mRNA and protein levels by RNA interference (P< 0.01). Proliferation was inhibited and drug resistance to gemcitabine was improved (P< 0.05). Flow cytometry showed that the proportion of cells in the G1/G0 phase increased (P< 0.01), and in G2/M and S phases decreased (P< 0.05), with no apparent differences in apoptosis (P> 0.05). The capacity for invasion was markedly reduced (P< 0.01). In addition, down-regulating Ezrin expression had no effect on phosphorylated-Akt protein (P>0.05), but could decrease the level of phosphorylated-Erk1/2 protein (P< 0.05). Conclusions: RNA interference of Ezrin could inhibit its expression in the pancreatic cancer cells line Panc-1, leading to a potent suppression of malignant behavior in vitro. Assessment of potential as a target for pancreatic cancer treatment is clearly warranted.

Keywords: Pancreatic cancer - Ezrin - RNA interference - cell cycle - proliferation - apoptosis - invasiveness

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Introduction

Ezrin encoded by the Vil2 gene, is a member of the ERM(ezrin-radixin-moesin) protein family due to the common membrane-binding N-terminal FERM domain with band-4.1 family members (Turunen et al., 1994). In resting cells, inactive Ezrin is retained in the cytoplasm in a closed conformation that masks transmembrane protein binding sites in their N-terminal domain (FERM) and F-acting-binding sites in the C-terminal region (Andréoli et al., 1994). The conformational masking is achieved by both intramolecular and intermolecular interactions (Gautreau et al., 2002). Ezrin could unfold into an active conformation when phosphatidylinositol-4,5-bisphosphate (PIP2) through its N-terminal or phosphorylation at conserved C-terminal.

Bing an"intracellular scaffolding", Ezrin is not only involved in the cytoskeletal organization but also involved in a number of signaling pathways (Louvet-Vallee et al., 2000). Furthermore recent evidence has shown Ezrin participates in regulating proton pumps and ion channels (Kurashima et al., 1999; Darmellah et al., 2009). Therefore, Ezrin protein play a crucial role in multiple cellular functions, comprising cell survival, adhesion, motility, phagocytosis and morphogenesis (Paglini et al., 1998; Lugini et al., 2003; Luciani et al., 2004; Jeon et al., 2009; Killock et al., 2009). These functions are of fundamental importance during early development and homeostasis of tissues and organs (Polesello et al., 2004; Saotome et al., 2004).

Ezrin is expressed in a variety of normal cells, including many types of epithelial, lymphoid and glial cells (Crepaldi et al., 1997; Wick et al., 2001; Mielgo et al., 2007), and exerts indispensability functions. Ezrin knockout mice died 21 days after birth because of restricted development of intestinal villi, a site in which other ERM proteins are not expressed (Saotome et al., 2004). But aberrant expression of Ezrin, especially over expression, has been found associated closely with malignant tumors (Bruce et al., 2007). Ezrin was over expression in melanoma and the level of expression was parallel with its thickness and invasive ability (Ilmonen

*[1]Department of General Surgery, [2]Department of Anesthesiology, Beijing Tiantan Hospital, Capital Medical University, Beijing, China *For correspondence: songmaomin@163.com*

et al., 2005; Federici et al., 2009). And there is a positive correlation between Ezrin overexpression and the metastatic potential of human breast cancer cells (Li et al., 2008). Suppression the over expression of Ezrin in rhabdomyosarcoma and osteosarcoma cells with high-metastatic potential could reduce their metastatic ability (Khanna et al., 2004; Yu et al., 2004). Not only that strong Ezrin immunoreactivity in tumor tissues correlate with a poor prognosis has been found in patients with so many malignancies, such as esophageal cancer (Chai et al., 2007; Zhai et al., 2010), gastric carcinoma (Shi et al., 2006), colorectal cancer (Elzagheid et al., 2008), astrocytic tumors (Geiger et al., 2000), highly malignant soft tissue sarcoma (Weng et al., 2005) and squamous cell carcinoma of the head and neck (Mhawech-Fauceglia et al., 2007). All these revealed Ezrin key role in malignant tumor cells migration and progression. Therefore sometimes Ezrin was depicted as an prognosis predictor of malignant tumor (Mäkitie et al., 2001).

To investigated the role of Ezrin in pancreatic cancer cell, we down-regulated the expression of Ezrin by RNA interference(RNAi). We conducted cellular proliferation, cycle, apoptosis, invasion and drug sensibility assays to elucidate the role of Ezrin in pancreatic cancer cell line Panc-1.

Materials and Methods

Antibodies and reagents

Rabbit polyclonal anti-ezrin antibody and HRP-conjugated secondary antibody(goat anti-rabbit IgG-HRP) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-phospho-AKT(Ser473), anti-phospho-p44/42 MAPK (Erk1/2) antibodies and HRP-conjugated secondary antibody(horse anti-mouse IgG-HRP) were purchased from Cell Signaling Technology (Beverly, MA, USA). The pSilencer2.1 U6 was purchased from Ambion (Austin, TX, USA). Gemcitabine was purchased from Eli Lilly (Indianapolis, IN, USA). The siRNA and DNA chains were synthesized by Shanghai GenePharma Co, Ltd (Shanghai, China).

Cell Culture

Human pancreatic cancer cells line Panc-1, obtained from Shanghai Institute of Biochemistry and Cell Biology, were maintained in DMEM (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) and were incubated in a humidified (37 °C, 5% CO_2) incubator, grown in 75 cm^2 culture flasks and passaged upon reaching 80% confluency.

Ezrin siRNA Preparation

Looked up human full-length sequences of Ezrin gene (GenBank: NM_001111077). Designed three pairs of siRNA correspond to:
No.1 273–291bp (ATCAGGTGGTAAAGACTAT)
No.2 312–330bp (ACTTTGGCCTCCACTATGT)
No.3 896–914bp (GGCTTTCCTTGGAGTGAAA).
siRNA-1:
5'-AUCAGGUGGUAAAGACUAUdtdt-3'(sense)
5'-AUAGUCUUUACCACCUGAUdtdt-3'(antisense)
siRNA-2:
5'-ACUUUGGCCUCCACUAUGUdtdt-3'(sense)
5'-ACAUAGUGGAGGCCAAAGUdtdt-3'(antisense)
siRNA-3:
5'-GGCUUUCCUUGGAGUGAAAdtdt-3'(sense)
5'-UUUCACUCCAAGGAAAGCCdtdt-3' (antisense)
A nonspecific 21-nucleotide siRNA was used as a control.
siRNA-NC:
5'-ACGUGCACCGUUCGGAGAAdtdt-3'(sense)
5'-UUCUCCGAACGGUGCACGUdtdt-3' (antisense

Transient Transfection and Screening

Transient transfections of siRNA were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as suggested by the manufacturer. The Panc-1 cell without siRNA was another control. Cells were assayed 48 and 72 hours after transfection by real-time quantitative PCR and Western blot to screen out the most efficient one.

Construction of plasmid vectors and transfection

Since the siRNA-1 was the most efficient one according to Real-time PCR and Western blot (Figure 1), we choes the sequences No.1 (273–291bp) as the target. Synthetized the DNA chains with BamH I and Hind III sticky end restriction sites and loop. (top)5'-GATCCAT CAGGTGGTAAAGACTATTTCAAGAGAATAGTCT TTACCACCTGATTTTTTTGGAAA-3' (Bot)5'-AGCT TTTCCAAAAAAATCAGGTGGTAAAGACTATTCT CTTGAAATAGTCTTTACCACCTGATG-3'

A pair of nonspecific DNA chains were used as the control. (top)5'-GATCCACTACCGTTGTTATAGGTGC TCAAGAGACACCTATAACAACGGTAGTAATTTTT TGGAAA-3' (Bot)5'-AGCTTTTCCAAAAAATTACTA CCGTTGTTATAGGTGTCTCTTGAGCACCTATAACA ACGGTAGTG-3'

The two pairs of DNA were annealed and inserted into the BamH I and Hind III sites of pSilencer 2.1-U6 and transformed into XL1-Blue competent cells (Promega, Madison, WI, USA). Positive clones were identified and verified by restriction enzyme analysis and sequence analysis.

The two plasmid vectors together with the vector without plasmid were transfected into Panc-1 cells by Lipofectamine 2000, named pSilencer2.1 Ezrin, pSilencer 2.1 NC and pSilencer2.1 vector. The Panc-1 cell with transfection reagents was another control.

Real-time quantitative PCR

The total cellular RNA was isolated from the collected cells by Trizol. The Ezrin mRNA copies were quantified using real-time quantitative PCR Kit (Takara) on a Bio-Rad iQ5 Sequence Detection System (Bio-Rad, Hercules, CA, USA). The primers of Ezrin and β-actin were as follows:
forward primer 5'-GGACTGATTGAATTACGG-3',
reverse primer 5'- AACAAGTATGGCACAGATG-3' and
forward primer 5'-CACCAACTGGGACGACAT-3',
reverse primer 5'-ATCTGGGTCATCTTCTCGC-3'.

The housekeeping gene β-actin was amplified to normalize the Ezrin mRNA expression. The copy numbers of β-actin and Ezrin were determined according to

each standard curve. Relative Ezrin mRNA levels were determined by comparing the PCR cycle thresholds between the cDNA of Ezrin and that of β-actin.

Western blotting assay

The cellular total proteins were extracted by RIPA Lysis Buffer (Takara) 72 h after transfection and 40 μg was added to each well for 100 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After semi-dry electric transfer at 100V for 1h, a hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Germany) was sealed with 50 g/L skim milk powder at room temperature (15-25 °C) for 1.5h, and incubated at 4 °C overnight with rabbit polyclonal anti-ezrin antibody, mouse monoclonal anti-phospho-AKT (Ser473), anti-phospho-p44/42 MAPK (Erk1/2) antibody and mouse anti-human β-actin monoclonal antibody respectively. After being washed, it was incubated at 37 °C for 2 h with second-antibody and colored by ECL. It was scanned for the relative value of protein expression in gray scale by Image-Pro plus software 6.0.

Cell proliferation assay

Cell proliferation was measured by MTT assay in 96-well micro-culture plates. Cells were collected 24 h after transfection, and plantd at 5×10^3 cells/well in 96-well plates in DMEM containing 10% FBS. Five duplicate wells were set up for each group and the test was repeated 3 times. Blank control cells served as control. After 24, 48, 72, 96, 120, 144, 168 hours of incubation, 20 μl of 5 mg/ml solution of MTT (Sigma Co, Ltd.) in PBS was added to each well for 4 hours. Absorbance of each well was read by a Bio-Rad 550 Microplate Reader (Hercules, CA) at a wavelength of 490 nm. Proliferation curves were plotted for each of the groups.

Flow Cytometry

After synchronized in serum-free medium(SFM) for 48h and cultured in complete medium for 24h, the cells were collected by trypsinization. After being washed twice with PBS, they were fixed with 70% cold alcohol for 24h at 4 °C. Prior to cell cycle analysis, cells were resuspended in PBS containing RNase A (20 ug/mL, Sigma) and 20 μg/ml propidium iodide (PI; Sigma), and then incubated at 37 °C for 30 min in the dark at room temperature before analyses for PI fluorescence intensity using the FACSCalibur flow cytometer (Becton Dickinson Labware, Franklin Lakes, US). A total of 10,000 events were counted for each sample group. The relative proportions of cells in the G_0/G_1, S and G_2/M phases of the cell cycle were analyzed using FlowJo software (Tree Star, Ashland, OR). To detect the apoptosis, cells were trypsinized, counted, washed twice in ice-cold PBS solution, and resuspended in 200 μl 1×binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$). Next, 10μl of Annexin-V-R-PE (R&D Systems Europe Ltd., Abingdon, UK) was added to 100 μl of cell suspension. After being held on ice in the dark for 20-30 minutes, 10 μl of 7-AAD (Sigma) and 380 μl of binding buffer were added before the analysis on the flow cytometer. 10000 cells events were counted for each sample group.

Invasion Assay

Cell invasion assay was performed using Transwell chambers (Corning Co, Ltd). After coated with FN (0.5 mg/ml 10 μl)and matrigel (50 μg/ml, BD Biosciences), 5×10^4 cells (100 μL) 72h after transfection were resuspended in serum-free medium and placed into the upper compartment of the Transwell chambers. The lower compartment of the chambers was filled with 200 μL endothelial cell medium (ECM, sciencell, USA) serum-containing medium. Incubated in the incubator at 37 °C for 24 h, wiped off nonmigratory cells on the upper surface of the filter with a cotton swab. Invasive cells that penetrated through the pores and migrated to the underside of the membrane were stained with 1% crystal violet solution for 15min after fixation with 4% paraformaldehyde. 5 random fields were counted with a light microscope at 200 × magnification, and the average cell number was determined.

Drug resistance assay

The cells of pSilencer2.1 Ezrin and pSilencer 2.1 NC were collected 48h after transfection and plantd at 5×10^3 cells/well in 96 well plates in DMEM containing 10% FBS. After incubated at 37 ºC and 5% CO_2 for 24 hours, the culture medium was replaced by complete medium containing 1000, 100, 10, 1, 0.1, 0.01 and 0 mg/l Gemcitabine (Eli Lilly, Indianapolis, USA) at a final volume of 200 μl. Five duplicate wells were set up for each consistency of each group. MTT method was performed after 48 and 72 hours. The inhibitory rates of cell growth were calculated according to absorbance (A) at 490 nm as follows: inhibitory rate= (1 – mean A of drug groups/mean A of no drug groups) ×100%. The IC50 values were calculated by non-linear regression analysis using SigmaPlot 4.01 software.

Statistical analysis

Each experiment was performed three times. All the data were analyzed by SPSS13.0. All results are expressed as means ± SD. All statistical analyses were performed using one-way ANOVA. And P value <0.05 was considered significant.

Results

Construction of Ezrin suppression plasmid vector

Three pairs of specific siRNA(siRNA-1, siRNA-2, siRNA-3) and the negative siRNA(siRNA-NC) were transiently transfected into Panc-1 cells respectively. Real-time quantitative PCR and Western-blotting showed that three pairs of specific siRNA suppressed the expression of Ezrin significantly ($P < 0.05$). And the siRNA-1 (No.1 273–291bp) was the most effective one. We refered to the sequence No.1 (273–291bp) as the target. DNA fragments synthetized according to the sequence were annealed and connected and transformed into XL1-Blue competent cells.

Restriction enzyme analysis and sequence analysis approved that the No.1 sequence and negative control sequence had been cloned into pSilencer2.1 U6 vectors successfully (Figure 1).

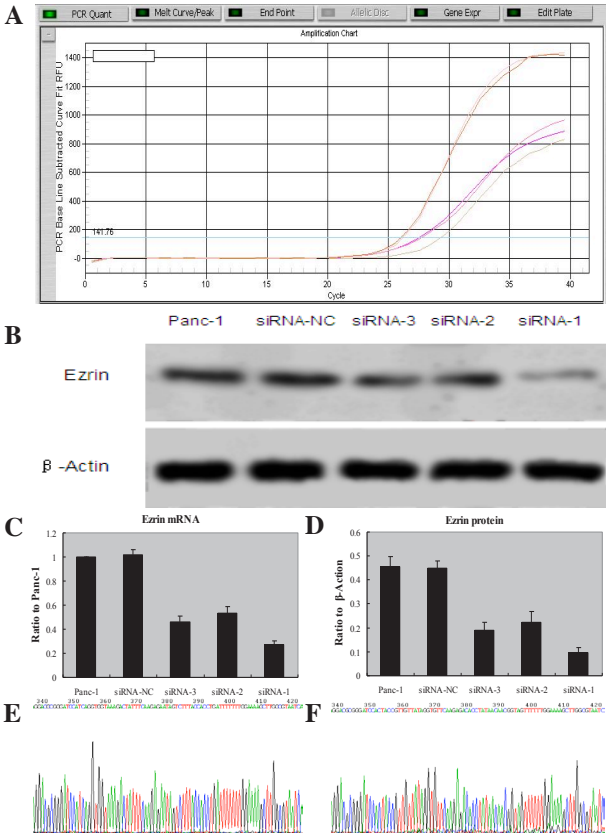

Figure 1. Selecting of siRNA and Construction of Plamid Targeting Ezrin. (A) Amplification Curve of real-time quantitative PCR. (B) Western blot showed the ezrin protein was suppressed after transient transfected specific siRNA. (C, D) Statistical analysis of real-time quantitative PCR and Western-blotting: Three pairs of specific siRNA suppressed the expression of ezrin significantly ($P < 0.05$). And the siRNA-1 (No.1 273–291bp) was the most effective one, shown by one-way ANOVA. (E, D)DNA sequencing of pSilencer2.1 Ezrin and pSilencer2.1 NC: DNA fragment according to the sequences of siRNA-1 and negative control had been cloned into pSilencer2.1 U6 vector

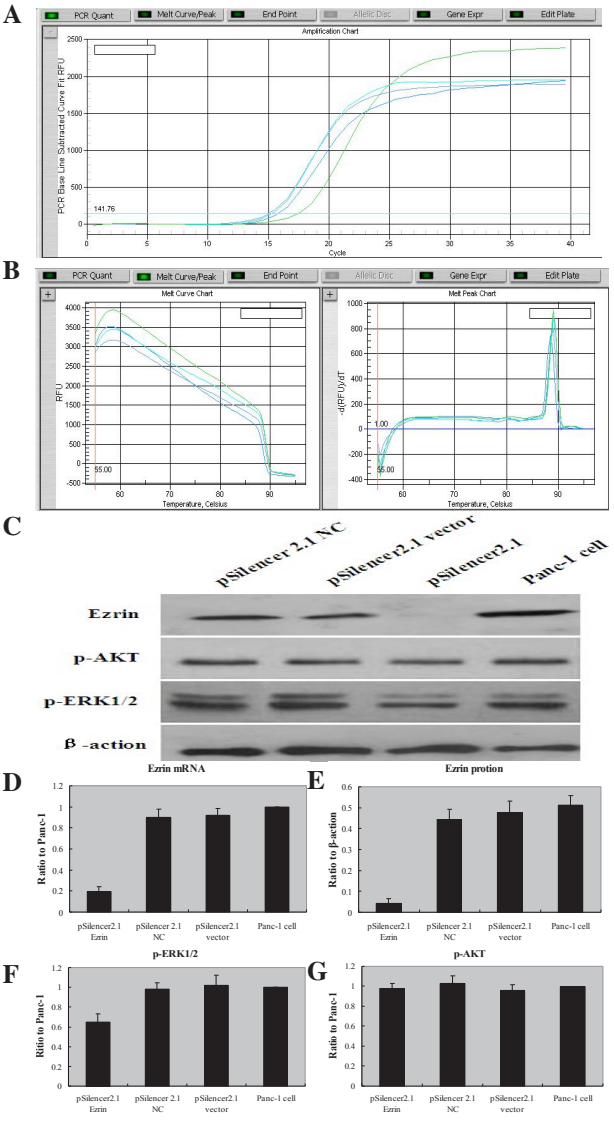

Figure 2. The Effects of Ezrin Silenced and Influence on Phosphorylated-Erk1/2 and Phosphorylated-Akt. (A) Amplification Curve of real-time quantitative PCR of Ezrin gene. (B) Solubility curve of real-time quantitative PCR of Ezrin gene. (C) Western blot of Ezrin, phosphorylated-Erk1/2 and phosphorylated-Akt protein. (D) Statistical analysis of real-time quantitative PCR: Compared to the three control groups (pSilencer 2.1 NC, pSilencer2.1 vector and Panc-1 cell), Ezrin mRNA of pSilencer2.1 Ezrin decreased 78.26%,78.8% and 80.5%. (E) Statistical analysis of Western-blotting: Compared to the three control groups , Ezrin protion of pSilencer2.1 Ezrin decreased 90.52%, 91.18% and 91.8%. (F, G) Statistical analysis of Western-blotting of phosphorylated-Erk1/2 and phosphorylated-Akt: Compared to the control, phosphorylated-Erk1/2 protion of pSilencer2.1 Ezrin decreased obviously ($P < 0.05$), and the level of phosphorylated- Akt protein had no obvious change ($P>0.05$)

Suppressing the expression of Ezrin and the effect on phosphorylated-Erk1/2 and phosphorylated-Akt

Plasmids (pSilencer 2.1 Ezrin, pSilencer 2.1 NC and pSilencer2.1 vector) were transfected into Panc-1 cells. 48 h and 72 h later, real-time quantitative PCR and Western-blotting were carried out. The results showed that compared with three control groups (pSilencer 2.1 NC, pSilencer2.1 vector and Panc-1 cell), wether Ezrin mRNA or Ezrin protein of pSilencer2.1 Ezrin decreased significantly ($P < 0.01$). However there was no remarkable difference among the three control groups ($P >0.05$) (Figure 2). Morever, our results indicated that down-regulating Ezrin expression decreased the level of phosphorylated-Erk1/2 protein ($P < 0.05$) but had no effect on phosphorylated-Akt ($P>0.05$), which suggest that the Erk1/2 pathway might participate in the Ezrin-mediated malignant behavior (Figure 2).

Ezrin silence retarding the growth of Panc-1 cell

Cell proliferation was monitored for 7 days after transfection. The results showed that there was no marked difference among the four groups cells at the first 24 h and 48 h ($P >0.05$). But the growth speed of pSilencer2.1 Ezrin began to be restrained significantly since 72 h after transfection($P < 0.05$). And the difference was more and more significant as time went on. However, the growth speed of the three control groups were in harmony throughout the experiment($P>0.05$) (Figure3). The result revealed that down-regulating Ezrin by RNA interference could restrain the proliferation of Panc-1 cell.

Effects on cell cycle and apoptosis

Flow cytometry was performed to observe the

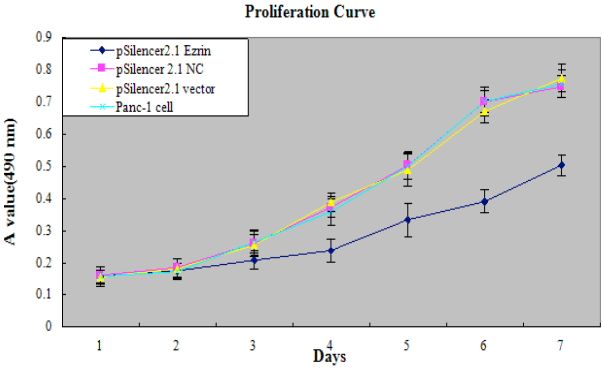

Figure 3. Effects of Ezrin on Panc-1 Cell Growth. The cell growth curves of the four groups were assayed on days 1-7: The growth speed of pSilencer2.1 Ezrin began to be restrained significantly since 72h after transfection ($P < 0.05$). And the difference was more and more significant as time went on. However, the growth speed of the three control groups were in harmony throughout the experiment ($P>0.05$)

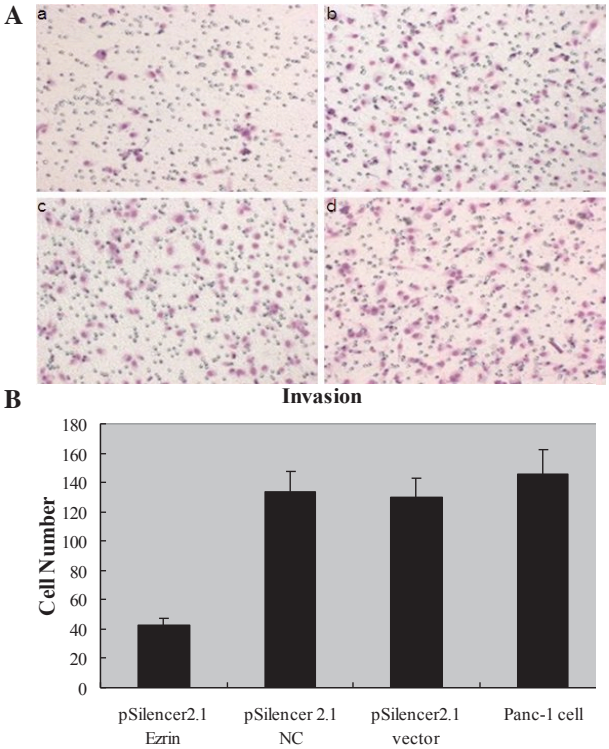

Figure 5. Effects on Cell Invasion. (A) Transwell chambers were used to detect cell invasion: The cells migrating to the lower chambers were observed with a light microscope at 200 × magnification. pSilencer2.1 Ezrin (a), pSilencer 2.1 NC (b), pSilencer2.1 vector (c), Panc-1 cell (d). (B) The cells invading to the lower chambers were analyzed: Compared to the three control groups, the invasion ability of the pSilencer2.1 Ezrin cells was greatly decreased ($P < 0.01$)

effects of Ezrin silence on cell cycle and apoptosis. We observed that down-regulating Ezrin exppression by RNA interference induced great changes on cell cycle. The percentage of cells in G_1/G_0 phase increased obviously ($P < 0.01$). And the percentages of cells in G2/M phase and S phase decreased significantly($P < 0.05$) (Figure4).

The effects of Ezrin silence on cell apoptosis were also inspected by flow cytometry. We found that the cellular apoptotic rates in the four groups were all extremely low (Figure 4). And no discrepancy was observed among them ($P >0.05$).

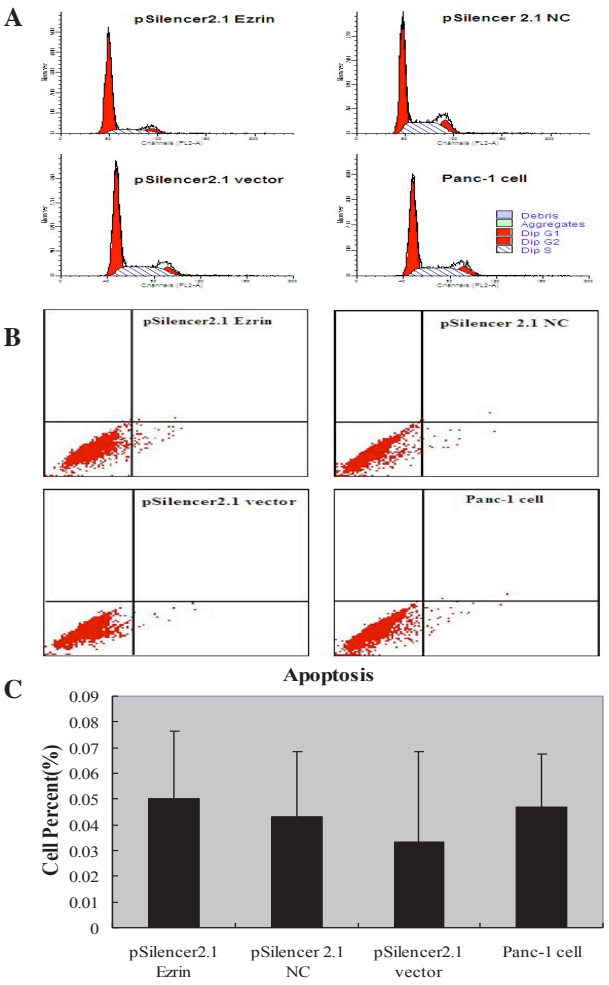

Figure 4. Detected Cell Cycle and Apoptosis by Flow Cytometer. (A, B) The effects of Ezrin silence on cell cycle: Down-regulating Ezrin exppression led to great changes in cell cycle. The percentage of cells in G1/G0 phase increased and percentages of cells in G2/M phase and S phase decreased obviously ($P < 0.05$). (C) Effects on cell apoptosis: The cellular apoptotic rates in the four groups were all extremely low. And no discrepancy was observed among them($P >0.05$)

Inhibition of invasion

Cell invasive ability was examined by transwell assay. The average cell number of 5 fields of the pSilencer2.1 Ezrin migrating to the lower chamber was (42.4±5.03) for each 200× field under the microscope. Compared to the three control groups pSilencer2.1 NC (133.6±14.05), pSilencer2.1 vector (129.8±13.05) and Panc-1 cell (145.6±16.79), the invasion ability of the pSilencer2.1 Ezrin cells was greatly decreased ($P < 0.01$). The results showed that Ezrin was involved in cell invasion and down-regulating Ezrin exppression could inhibit Panc-1 cell invasion (Figure 5).

Improving drug sensibility

IC50 means half maximal inhibitory concentration. And it is a measure of the effectiveness of a drug in inhibiting biological or biochemical function. To inspect whether Ezrin participate in drug-resistant to Gemcitabine in Panc-1 cell, we conducted the drug experiment. In the experiment, the IC_{50} at 48 h and 72 h were (7.90±0.43) mg/L and (5.20±1.26) mg/L respectively in pSilencer2.1 Ezrin. Compared to the pSilencer 2.1 NC (10.30±1.09)

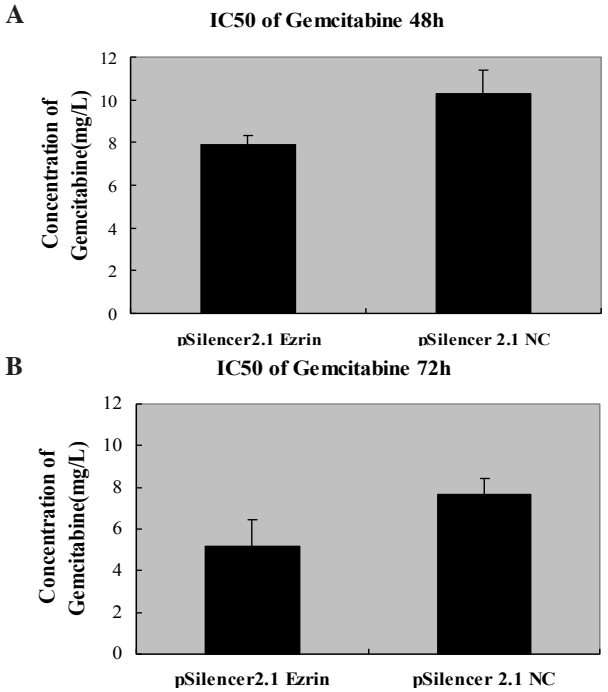

Figure 6. Effects on Drug Sensibility. (A) The Gemcitabine IC50 for pSilencer2.1 Ezrin cells at 48h was (7.90±0.43) mg/L, lower than pSilencer 2.1 NC(10.30±1.09) mg/L ($P < 0.05$). (B) The Gemcitabine IC50 for pSilencer2.1 Ezrin cells at 72h was (5.20±1.26)mg/L, lower than pSilencer 2.1 NC(7.66±0.75) mg/L ($P < 0.05$)

mg/L and (7.66±0.75) mg/L, the IC_{50} of Gemcitabine descend markedly ($P < 0.05$). These results suggest that Ezrin might participate in drug-resistant to Gemcitabine in Panc-1 cell, and down-regulating Ezrin exppression might improve the drug sensibility.

Discussion

Ezrin encoded by the Vil2 gene, is a member of the ERM (ezrin-radixin-moesin) protein famiy, which acts as membrane organizer and linker between the plasma membrane and cytoskeleton (Swanson et al., 2007; Fadiel et al., 2008). Since Ezrin involved in the cytoskeletal organization and a number of signaling pathways (Louvet-Vallee et al., 2000), it participates in the regulation of cell survival, adhesion, proliferation, migration and phagocytosis processes (Paglini et al., 1998; Lugini et al., 2003; Luciani et al., 2004; Jeon et al., 2009; Killock et al., 2009), which are fundamental importance during early development and homeostasis of tissues and organs (Polesello et al., 2004; Saotome et al., 2004).

Although Ezrin is expressed in a variety of normal cells (Crepaldi et al., 1997; Wick et al., 2001; Mielgo et al., 2007), but aberrant expression, especially over expression, has been found in vast majority of malignant tumors. Previous studies have reveal that the level of Ezrin expression is positively related to the degree of malignancy in many tumors (Ilmonen et al., 2005; Li et al., 2008; Federici et al., 2009). And over expression of Ezrin usually correlates with a poor prognosis. It has also been found that down-regulating the level of Ezrin expression could reduce the metastatic ability of rhabdomyosarcoma and osteosarcoma (Khanna et al., 2004; Yu et al., 2004). All the evidences indicate that Ezrin may play an important role in the tumorigenesis, development, invasion and metastasis process in malignant tumors.

Pancreatic cancer is one of the most aggressive tumors with extremely poor prognosis. Evidence has shown that Ezrin is high-expressed in pancreatic cancer tissues (Torer et al., 2007; Duan et al., 2008). And high-level Ezrin expression in pancreatic adenocarcinoma cell lines is associated with high metastatic potential (Akisawa et al., 1999). All these suggest that Ezrin may be a key regulatory molecule in development and progression of pancreatic cancer.

There is increasing evidence regarding the role of Ezrin in cell proliferation. Most of these studies, involving cancer tissues and cell lines, have reported direct relationships between cell proliferation and the level of Ezrin expression or phosphorylation in both malignant (Chen et al., 2001; Ohtani et al., 2002) and nonmalignant cells (Crepaldi et al., 1997). Although the mechanism is not entirely clear, so many factors have been found involved in Ezrin affecting cell proliferation. Extracellular signals such as the hepatocyte growth factor (HGF), the epidermal growth factor (EGF), or the platelet-derived growth factor (PDGF) induce phosphorylation of Ezrin in epithelial cells through stimulation of their transmembrane receptors (Fazioli et al., 1993; Gautreau et al., 1999). Furthermore Ezrin can be also activated by intracellular signaling factors such as the Src kinase Lck in T lymphocytes or the Src and Rho pathways in epithelial cells (Matsui et al., 1998; Autero et al., 2003; Srivastava et al., 2005).

Cell proliferation as a consequence of cell cycle progression is the key process that leads to clonal expansion of initiated cells during tumor promotion. Cyclin D1 is a cell cycle regulatory protein that acts as a growth factor sensor to integrate extracellular signals with the cell cycle machinery, particularly during the G_1 phase of the cell cycle (Haydar et al., 2011). Activation of the PI 3-kinase/AKT and/or ERK1/2 pathways can lead to cell proliferation and growth by regulating the cyclin D1 level (Mirza et al., 2000; Segrelles et al., 2006).

Extracellular signal regulated kinases1/2 (ERK1/2) or p44/42 mitogen-activated protein kinases (p44/42 MAPK) belong to a highly conserved family of Ser-Thr protein kinases and were characterized to function through the Ras-Raf-MEK-ERK1/2 cascade. ERK1/2 are involved in wide cellular processes, such as proliferation , differentiation and survival. ERK1/2 are widely expressed in all tissues and cell, and could be activated by multiple stimuli, such as epithelial growth factor (EGF) (Pearson et al., 2001). Overexpression or constitutive activation of ERK1/2 pathway can lead to progression of many cancers (Sebolt-Leopold et al., 2004).

AKT, a serine/threonine protein kinase, also known as protein kinase B and a member of AGC family, regulates a variety of cellular processes including proliferation, survival, migration and differentiation (Manning et al., 2007). Several lines of evidence demonstrate that Akt is a critical player in tumor development. Hyperactivation of the Akt pathway has been detected in up to 50% all human

tumors (Sun et al., 2001; Altomare et al., 2005) and is closely associated with chemoresistance (Xu et al., 2011). Previous experiments demonstrate that phosphorylation of Ezrin on residue Y145 promotes cell proliferation (Alexis et al., 1999), whereas phosphorylation on residue Y353 could induce the activation of the PI3K/Akt pathway, providing a mechanism for the function of Ezrin in cell survival (Gautreau et al., 1999; Autero et al., 2003).

In this study, we observed that although without effect on apoptosis, down- regulating the expression of Ezrion by RNA interference restrained the proliferation and changed the cell cycle, which retarded the growth of pancreatic cancer cell line Panc-1 in vitro. Not only that, according to the result of phosphorylated Erk1/2 and phosphorylated-Akt, we confer that the Erk1/2 pathway might participate in the Ezrin-mediated cell proliferation.

Experiments have shown a direct relationship between Ezrin expression and tumor migration and invasion. Ohtani et al. (1999) demonstrated that neoplastic cell migration was inhibited by Ezrin antisense oligonucleotides in endometrial cancer. Wick et al. (2001) found that glioma cell invasion could be blocked using dominant negative Ezrin expression constructs. Although the mechanism of Ezrin participating in metastasis in tumors is not entirely clear, Ezrin has been found involved in multiple metastatic pathways. For example, Ezrin interacts with the cell surface receptor CD44 to promote invasiveness (Martin et al., 2003), and incites metastasis via Rho activation (Yu et al., 2004). Ezrin plays a role in metastasis-associated cell-adhesion functions through interactions with E-cadherin (Mangeat et al., 1999). Ezrin also influences cell adhesion and migration as a downstream target of Src (Srivastava et al., 2005), and as a direct target of MET, a receptor tyrosine kinase inevitablly implicated in metastatic behavior (Bladt et al., 1995). Furthermore, The invasion of cells into the surrounding tissue is a complex process that requires cell-cell contact, cell motility and degradation of the extracellular matrix by matrix metalloproteinases (Deryugina et al., 1998; Stylli et al., 2008). Being a cytoskeletal protein, Ezrin might affect the assembly of cytoskeleton, which would facilitate cell migration and invasion. Our results were consistent with these previous reports in that down-regulating the level of Ezrin expression markedly inhibited the migration and invasion of Panc-1 cells in vitro. Considering the changes in phosphorylated ERK1/2 expression, we proposed the Erk1/2 pathway may also participate in Ezrin-induced cell motility and invasion in pancreatic cancer.

High resistance to chemotherapy is considered a common phenomenon and one of the major reasons for poor prognosis in pancreatic cancer. However the mechanism of tumor cells chemoresistance to chemotherapy is so complex, and so many factors participate in the process, such as checkpoint kinase 1 (CHK1), multidrug resistance protein5 (MRP5), focal adhesion kinase(FAK), nuclear factor NF-kappa-B(NF-κB) (David et al., 2009; Wu et al., 2009; Wolfgang et al., 2010; Min-Kyoung et al., 2011). The multidrug resistant (MDR) phenotype exists widely in many tumor cells, and it is often associated with an increased expression of P- glycoprotein at the plasma membrane (Juranka et al., 1989). Luciani (Luciani et al., 1995) found that P-glycoprotein colocalized and coimmunoprecipitated with Ezrin, and treatment with antisense oligonucleotides for Ezrin could restore drug susceptibility in lymphoid origin cells. In addition, it has been found that activate the Raf/MEK/ERK pathway may increase the levels of the Mdr-1 drug pump and the anti-apoptotic Bcl-2 protein. And the increased expression of Mdr-1 and Bcl-2 is associated with the drug resistance of breast cancer cells (Weinstein-Oppenheimer et al., 2001; Davis et al., 2003). In our experiment, after down-regulating Ezrin expression, the half maximal inhibitory concentration (IC_{50}) of Gemcitabine to Panc-1 cells decreased significantly.

The result shows that Ezrin may participate in the complex process of drug resistance, and inhibition on Ezrin expression could increase the drug sensibility to Gemcitabine in Panc-1 cells.

In conclusion, we successfully silenced the Ezrin expression in Panc-1 cells by RNA interference, and found that decreasing the Ezrin expression were correlated with cell proliferation, cycle, invasion and drug sensibility to Gemcitabine. Based on these results, we propose that Ezrin play a critical role in pancreatic cancer progression, and ERK1/2 pathway may be involved in this role. These results indicate that blocking Ezrin function may represent a novel and effective strategy for preventing pancreatic cancer progression, invasion and metastasis.

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