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Downregulation of integrin-linked kinase inhibits epithelial-to-mesenchymal transition and metastasis in bladder cancer cells

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ABSTRACT

Integrin-linked kinase (ILK) is a multifunctional serine/threonine kinase in cytoplasm. Recent studies showed that cancer patients with increased ILK expression had low survival, poor prognosis and increased metastasis. Although the causes of ILK overexpression remain to be fully elucidated, accumulating evidence suggests that its oncogenic capacity derives from its regulation of several downstream targets that provide cells with signals that promote proliferation, survival and migration. However, the mechanisms underlying tumor metastasis by ILK is still not fully understood. Epithelial–mesenchymal transition (EMT) is a critical event of cancer cells that triggers invasion and metastasis. We recently reported that knockdown of ILK inhibited the growth and induced apoptosis in human bladder cancer cells. Therefore, we postulate that ILK might involve in EMT. Here we further investigate the function of ILK with RNA interference in bladder cancer cells. Knockdown of ILK impeded an EMT with low Vimentin, Snail, Slug and Twist as well as high E-cadherin expression in vivo and vitro. In addition, we found that knockdown of ILK inhibited cell proliferation, migration and invasion as well as changed cell morphology, adhesion and rearranged cytoskeleton in vitro. We also demonstrated that ILK siRNA inhibited phosphorylation of downstream signaling targets Akt and GSK3β, increased expression of nm23-H1, as well as reduced expression of MMP-2 and MMP-9 in vivo and vitro. Furthermore, downregulation of ILK could increase expression of Ribonuclease inhibitor (RI), an important acidic cytoplasmic protein with many functions. Finally, the effects of ILK siRNA on bladder cancer cell phenotype and invasiveness translate into suppression for tumorigenesis and metastasis in vivo. Taken together, our findings highlight that ILK signaling pathway plays a novel role in the development of bladder cancer through regulating EMT. ILK could be a promising diagnostic marker and therapeutic target for bladder cancer.

1. Introduction

The bladder cancer is a very common malignant tumor at the urinary system with late aggravation and easy recurrence. According to statistics of the American Cancer Society, bladder cancer is the fourth most common cancer and is the ninth leading cause of death from cancer. Unfortunately, bladder cancer remains one of the least studied cancers worldwide, and up to now, the molecular mechanisms and signaling pathways involved in the initiation, promotion, and progression of bladder cancer are not fully understood [1,2].

ILK, a cytoplasmic effector of integrin receptors, is a widely expressed serine/threonine protein kinase located in focal adhesions (FAs). ILK is a central component of signaling cascades that control an array of biological processes that are crucial both to normal tissue homeostasis and to the progression of malignant disease. Activation of ILK by integrins and soluble mediators results in the regulation of downstream effectors that, in turn, modulate processes such as motility and contractility, survival, invasion, proliferation, and angiogenesis [3,4]. In addition, ILK is a downstream substrate of phosphoinositide 3-kinase (PI3K), and an important upstream kinase for the regulation of protein kinase B (PKB/Akt) and GSK3β [5]. ILK overexpression has been shown to promote invasion and cell migration. ILK expression and activity are increased in many types of cancer, such as prostate, colon, gastric and ovarian cancers, malignant melanomas, malignant pleural mesothelioma and NSCLC [6–8]. Our recent studies showed that siRNA targeting ILK could inhibit the growth and induce apoptosis in human bladder cancer cells. However, the mechanisms of ILK in bladder cancer invasion and metastasis remain unknown so far, especially how ILK regulates associated signaling pathway to affect tumor metastasis is not fully clear.

The epithelial-to-mesenchymal transition (EMT) is a physiologic process, originally described in embryonic development, in which cells lose epithelial characteristics and gain mesenchymal properties. Recently, EMT has gained attention as a critical phenotypic alteration of cancer cells to acquire invasive and metastatic ability [9]. EMT is

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mediated through several transcription repressors, such as Snail, Slug, Twist and ZEB1, and these EMT inducers typically suppress the transcription of the E-cadherin gene, an epithelial cell marker and a potent suppressor of tumor cell invasion and metastasis [10,11].

Therefore, we postulated that ILK signaling pathway might be involved in EMT of the acquisition of invasiveness and metastatic ability in bladder cancer. In this study, we stably knocked down ILK in human bladder cancer cell EJ and BIU-87 cells with RNA interference and ascertained whether siRNA ILK has any suppressive effects on the EMT and metastasis of these cells. We also explored the involvement of various molecular targets in this process. We present evidence that knockdown of ILK inhibited bladder cancer proliferation and invasion, changed cell morphology, more importantly, suppressed bladder cancer EMT as well as tumor growth and metastasis. In total, the data demonstrate a novel role for ILK signaling pathway in tumor development and EMT of bladder cancer cells. Our results suggest that ILK could be a potential therapeutic target for metastatic bladder cancer.

2. Materials and methods

2.1. Cell lines, animal and reagents

pGensil-1siRNA plasmid was obtained from Wuhan Genesil Biotechnology Co., Ltd.(Wuhan, PR China). BALB/C nude (nu/nu) mice were purchased from Peking University Laboratory Animal Center (Beijing, PR China). EJ cells were from KGI Biotechnology Development Co, Ltd. (Nanjing, PR China); BIU-87 cells were from Chinese Type Culture Collection (Wuhan, PR China). Fetal calf serum was from Hyclone (Logan, Utah, USA). RPMI 1640 medium and G418 were products of Gibco-BRL (Carlsbad, CA, USA). Lipofectamine 2000 reagent and Trizol were bought from Invitrogen, Inc., (Carlsbad, California). Monoclonal primary mouse antibody of anti-human ILK, rabbit anti-human β-actin and CD31 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal primary rabbit antibody of anti-human p-Akt, p-GSK3β, MMP-2,MMP-9, nm23-H1, E-cadherin, Snail, Slug, Vimentin and Twist were purchased from Bioworld Technology, Inc (St. Louis, USA). The rest of the primary antibodies are from Beijing Zhongshang Biotechnology (Beijing, PR China).

2.2. Construction of ILK siRNA expression plasmids

Human ILK cDNA sequence (accession number: NM_004517) was provided by the GenBank. Appropriate sense strands of oligonucleotides that target the ILK mRNA siRNA were designed using ambion online siRNA finder (www.ambion.com). A random, with no homology to the human sequence was used as non-targeting control; it contains the same oligonucleotides with ILK siRNA. ILK siRNA expressed plasmids were constructed as follows: ILK siRNA forward and reverse:

5′-GATCCGGAAGAGCAGGGACTTCAATTCAAGAGATT-GAAGTCCCTGCTCTTCCTTTTTTGTCGACA-3′
3′-GCCTTCTCGTCCCTGAAGTTAAGTTCTCTAACTTCAGGGACGAGAA-GGAAAAAACAGCTGTTCGA-5′

siRNA control forward and reverse:

5′-GATCCGAGACTACAGTCTCGATCCTTTCAAGACGAGGATCGA-GACTGTAGTCTTTTTTGTCGACA-3′
3′-GCTCTGATGTCAGAGCTAGGAAAGTTCTGCTCCTAGCTCTGACATC-AGAAAAAACAGCTGTTCGA-5′.

Annealed double-stranded oligonucleotides were connected into the pGensil-1 plasmid. All recombinant plasmids were identified by endonuclease Sal I digesting. Finally they were further verified by DNA sequencing.

2.3. Cell culture and gene transfection

The EJ and BIU-87 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in a 5% CO_2 incubator at 37 °C. Twenty-four hours before transfection, cells were seeded into 6-well plates with an antibiotic-free medium at a density of 2×10^5 per well. The cells were transfected with the plasmids using Lipofectamine 2000 reagent according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were maintained in the presence of medium containing 800 ug/ml of G418 for 14 days and 400 μg/ml of G418 for additional 2 weeks. The individual G418-resistant monoclines were proliferated, and named EJ siILK or BIU-87 siILK cells and EJ vector or BIU-87 vector cells, respectively. The expression of knockdown ILK was detected for each clone by fluorescence microscope, RT-PCR and Western blotting.

2.4. RT-PCR analysis

Total RNA was isolated from cells using Trizol reagent (Invitrogen). Reverse transcription was performed and cDNAs were amplified with the following primer pairs, ILK forward: 5′-GCC AGG CTG TGA AGT TTG C-3′and reverse: 5′-TGC TGA GCG TCT GTT TGT G-3′; GAPDH of the same sample was used as an internal control, GAPDH forward: 5′-GCT GTC CCT GTA CGC CTC TG-3′ and reverse: 5′-TGCCGATGGTGATGACCTGG-3′. RT-PCR was conducted with the following parameters: 37 °C for 15 min, 85 °C for 5 s for RT reaction, then 94 °C for 2 min, 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min and a total of 30 cycles, then a final extension of 72 °C for 10 min. The PCR products were loaded on a 2% agarose gel, stained with GoldView, photographed under UV illumination. Results were collected and analyzed with MJ Opticon Monitor Analysis Software (Bio-Rad). Experiments were performed in triplicate and repeated three times.

2.5. Western blot assay

The EJ and BIU-87 cells' total proteins were extracted using cell lysis buffer, the protein concentration was measured using Enhanced BCA Protein Assay Kit. Equal amounts (20 μg) of protein were loaded into each lane. The samples were separated by the electrophoresis in a 10% SDS-PAGE gel electrophoresis, and then were electrotransferred to a PVDF membrane at 200 mA for 1.5 h. Next, the samples were blocked with 3% bovine serum albumin in TBST buffer overnight at 4 °C. The membrane was probed with antibodies against ILK (1:200 dilution), MMP-2, MMP-9, nm23-H1, E-cadherin, Snail, Slug, Vimentin, p-AKT and p-GSK3β (1:500 dilution) and mouse anti-actin primary antibody (1:3000 dilution) respectively for 2 h at 37 °C, washed thoroughly for 3×10 min with TBST, and incubated with goat anti-mouse secondary IgG (1:2000 dilution) and goat anti-rabbit secondary IgG (1:2000 dilution) respectively for 1 h at 37 °C, washed thoroughly 3×10 min with TTBS. The bands were detected by enhanced chemiluminescence method (BeyoECL Plus). Results were collected and analyzed with MJ Opticon Monitor Analysis Software (Bio-Rad). Experiments were performed in triplicate and repeated three times.

2.6. Immunofluorescence and laser scanning confocal detection

The cells were incubated for 24 h on cover slips in 6-well plates and washed with PBS, fixed with ice-cold 80% acetone for 10 min. The cover-slips were treated with 3% BSA in PBS for 30 min at 37 °C to block non-specific antibodies. Then they were incubated in antibodies against ILK (1:50 dilution), p-Akt (S473) (1:300 dilution), p-GSK3β (S9) (1:300 dilution) and RI (1:300 dilution) overnight at 4 °C. After they were washed three times with PBS, cells were incubated with secondary antibody DyLightTM 594-Conjugated Goat Anti-Mouse IgG (1:100 dilution), Rhodamine-Conjugated Goat Anti-Rabbit IgG (1:100 dilution) and TRITC-Conjugated Goat Anti-Rabbit