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Down-regulating ribonuclease inhibitor enhances metastasis of bladder cancer cells through regulating epithelial–mesenchymal transition and ILK signaling pathway

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ABSTRACT

Accumulating evidences implicate that ribonuclease inhibitor (RI) plays a suppressing role in cancer development. However, the mechanisms underlying antitumor of RI remain largely unknown. Epithelial–mesenchymal transition (EMT) is regarded as a key event in tumor progression. The reports have demonstrated that EMT was implicated in metastasis of bladder cancer. Therefore, we suppose that RI might involve regulating EMT of bladder cancer. Here bladder cancer T24 cells were transfected with pGensil-1-siRNA-RI vectors. HE staining, living cell observation, Phalloidine-FITC staining of microfilament, cell adhesion, scratch migration, and Matrigel invasion were examined respectively. RI expression and colocalization with ILK were detected using confocal microscope. Proteins associated with EMT were determined with Western blotting and immunohistochemistry in vivo and in vitro. Effects of RI expression on tumor growth, metastasis and EMT related proteins in BALB/C nude mouse and clinical human bladder cancer specimens were valued with histological, immunohistochemical and immunofluorescent examination respectively. We demonstrated that down-regulating RI increased cell proliferation, migration and invasion, changed cell morphology and adhesion, and rearranged cytoskeleton by inducing EMT and ILK signaling pathway in bladder cancer cells. In addition, we showed that down-regulating RI promoted tumorigenesis and metastasis of bladder cancer in vivo. Finally, we found that bladder cancer with invasive capability had higher Vimentin, Snail, Slug and Twist as well as lower E-cadherin and RI expression in clinical human specimens. Our results suggest that RI could play a novel role in inhibiting metastasis of bladder through regulating EMT and ILK signaling pathway.

Introduction

Bladder cancer is the most common tumor in the urinary system. Yearly almost 400,000 new cases of urinary bladder cancer are diagnosed in the world and more than 150,000 people die of the disease. In the US, bladder cancer is the fifth most frequent malignancy and the most expensive tumor to treat (Jacobs et al., 2010; Szepeshazi et al., 2012).

Epithelial-to-mesenchymal transition (EMT) is the process by which epithelial cells dramatically alter their shape and motile behavior as they differentiate into mesenchymal cells. EMT is typically characterized by the loss of the epithelial marker E-cadherin and increased expression of EMT-associated transcription repressors, such as Snail, Slug, Twist and ZEB1 (Schulte et al., 2012). Accumulating evidences show that EMT, to a great extent, is involved in invasion and metastasis of bladder cancer (McConkey et al., 2009; Tran et al., 2013).

Human ribonuclease inhibitor (RI), a cytoplasmic acidic protein with molecular weights of 50 kDa, contains 32 cysteine residues and consists of 15 leucine-rich repeats (LRRs). Such repeats have been identified in more than 100 proteins that exhibit a wide range of functions, including cell-cycle regulation, DNA repair, extracellular matrix interaction, and enzyme inhibition. The RI recognizes and inhibits ribonucleases by affinity (Shapiro, 2001). Yet, the biological role of RI is not known in its entirety (Nekrasov and Zinchenko, 2010). According to the structural genomics, we suppose that RI might be implicated in other unknown biological functions. Recently, we reported that up-regulating RI could inhibit melanoma growth, EMT and metastasis (Pan et al., 2012). We previously found that down-regulating RI could significantly promote growth of non-invasive bladder cancer BIU-87 cells (Chen et al., 2011).

Integrin-linked kinase (ILK), a central component of signaling cascades, controls an array of biological processes such as motility and contractility, survival, invasion, proliferation, and angiogenesis (McDonald et al., 2008). There were evidences that ILK was complicated

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in EMT of bladder cancer (Matsui et al., 2012). We lately reported that ILK siRNA inhibited EMT, metastasis and growth in bladder cancer cells; meanwhile, the experiment showed that RI and ILK had a colocalization and negative expression correlation in cytoplasm, which suggests that RI and ILK might have a certain interaction or relevant function (Zhu et al., 2012).

However, whether RI plays a significant role in inhibiting development of bladder cancer and its biological mechanisms remains fully unknown so far. According to the above researches, hypothetically, we focused on regulating EMT and ILK signaling pathways by RI in bladder cancer. Here, We found that knockdown of RI not only changed cell morphology, increased cell proliferation and invasion, but also promoted EMT as well as tumor growth and metastasis. Our findings elucidate that RI might have a metastasis suppressor function in bladder cancer by regulating EMT and ILK signaling pathway. RI could be a novel potential target for therapy of bladder cancer.

Materials and methods

Materials

T24 cells, pGenesil-1 siRNA plasmids were prepared by our laboratory. Appropriate sense strands of oligonucleotides that target the RI mRNA siRNA were designed using Ambion online siRNA finder (www.ambion.com). A random, with no homology to the human sequence (siRNA1) was used as control. BALB/C nude (nu/nu) mice were bought from Peking University Laboratory Animal Center (Beijing, PR China). Animals were bred in the Laboratory Animal Center and all studies were performed in agreement with the local ethics committee. Bladder tumor tissue samples of human were from The First Affiliated Hospital of Chongqing Medical University with informed consent and with the approval of the institutional ethics committee. RPMI 1640 medium and G418 were from Gibco-BRL (Carlsbad, CA, USA). Fetal calf serum was from HyClone (Logan, UT, USA). Lipofectamine 2000 reagent and TRIzol were purchased from Invitrogen, Inc. (Carlsbad, CA, USA). The monoclonal mouse anti-human ILK antibody and rabbit anti-human CD31 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit anti-human antibody of RI was kept by our laboratory. Monoclonal rabbit anti-human antibody of p-Akt, p-GSK3β, E-cadherin, N-cadherin, MMP-2, MMP-9, Vimentin, Cyclin-D1, Snail, Slug and Twist were purchased from Bioworld Technology, Inc. (St. Louis, USA). Goat anti-rabbit and goat anti-mouse antibodies of β-actin were purchased from Beijing Zhongshan Biotechnology (Beijing, PR China).

RI-siRNA vector construction

RI-siRNA expressed plasmids were constructed following a length of palindrome sequence (19 nucleotides sense strand and antisense strand) separated by a 9 nucleotide spacer following 4 continuous thymines as terminate signal, and three restriction enzyme sites (Sal I, BamH I and Hind III) located at two termination ends. Target sites for RI knockdown were selected (accession number NM_002939), and appropriate sense strands of oligonucleotides that target the RI mRNA were designed using Ambion online siRNA finder (www.ambion.com). The two target sequences selected (named siRNA2 and siRNA3) 5′-GATCCGAGGCATGAAGAACTCTTGATTCAAGACGTCAAGAGTTCTTCATGCCTTTTTTTGTCGACA-3′; 3′-GCTCCGTACTTCTTGAGAACTAAGTTCTGCAGTTCTCAAGAAGTACGGAAAAAAACAGCTGTTCGA-5′ and 5′-GATCCGGACATCAGCTCTGCACTTTTCAAGACGAAGTGCAGAGCTGATGTCCTTTTTTTGTCGACA-3′; 3′-GCCTGTAGTCGAGACGTGAAAAGTTCTGCTTCACGTCTCGACTACAGGAAAAAACAGCTGTTCGA-5′.

As a non-targeting control, a random sequence containing the same base composition was designed, moreover, no homology sequence with human. A length of 70 bps complementary DNA sequence was synthesized (named siRNA1: 5′-GATCCGAGAGACTACAGTCTCGATCCTTTCAAGACGAGGATCGAGACTGTAGTCTCTTTTTTTGTCGACA-3′; 3′-GCTCTCTGATGTCAGAGCTAGGAAAGTTCTGCTCCTAGCTCTGACATCAGAGAAAAAAACAGCTGTTCGA-5′).

Annealed double-stranded oligonucleotides were ligated into the pGenesil-1 plasmid. All recombinant plasmids were identified by endonuclease Sal I and Pst I digestion, which would respectively generate two bands and one band when the plasmids were constructed correctly. Finally they were further verified by DNA sequencing.

Cell culture and gene transfection

T24 cells were cultured in RPMI 1640 medium with 10% fetal calf serum, 100 mg/ml penicillin and 100 mg/ml streptomycin at 37 °C in a 5% CO_2 incubator. The cells were seeded into 6-well plates at a density of 2×10^5 cells/well for 24 h before transfection. The cells were transfected with pGensil-1 siRNA1-RI, pGensil-1 siRNA2-RI and pGensil-1 siRNA3-RI plasmids respectively using Lipofectamine 2000 reagent. Forty-eight hours after transfection, 400 μg/ml of G418 was used for screening positive clones for 14 days and 200 μg/ml of G418 for additional 2 weeks. The cells were named T24 siRNA1, T24 siRNA2, T24 siRNA3, T24 vector and T24 cells, respectively.

RT-PCR analysis

The total cellular RNA was isolated from the T24 cells using the TRIzol reagent according to manufacturer's instruction. cDNA was obtained by using PrimeScript RT reagent kit (TaKaRa, PR China), and then used TaKaRa LA Taq (TaKaRa, PR China) to amplified with the following primer pairs: RI forward: 5′-TCA GCG ACA ACC TCT TGG G-3′ and reverse: 5′-CAC AAT GCC GCA CAG GTC-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′-TGC CGA TGG TGA TGA CCT GG-3′. RT-PCR was conducted with the following parameters: 94 °C for 3 min for RT reaction, then 94 °C for 30 s, 59 °C for 30 s, 72 °C for 75 s and a total 30 cycles, then a final extension of 72 °C for 10 min. The PCR products were loaded on a 1% agarose gel, stained with GoldView and 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.

Immunofluorescence and laser scanning confocal detection

Cells were seeded on cover slips in 24-well plates and fixed by cold acetone for 20 min. then washed with PBS for 3×5 min. Cells were incubated with 3% BSA in PBS for 30 min at room temperature to block non-specific antibodies. Cells were incubated with RI (1:200; self-made), ILK, p-GSK3β and p-AKT (1:100 dilution; Bioworld, St. Louis, USA) antibodies overnight at 4 °C respectively. After washed three times with PBS, cells were incubated with secondary antibody DyLightTM 594-conjugated goat anti-mouse IgG, Rhodamine-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-rabbit IgG for 1 h at 37 °C respectively. Observations were performed under Olympus multifunction microscope (Tokyo, Japan) and Laser Scanning Confocal Microscope (Leica TCS-SP2, German).

Cytoskeleton, cell morphology and cell proliferation examination

Cells were seeded on cover slips in 24-well plates for 24 h and fixed by 4% paraform for 20 min, then blocked with BSA for 20 min, the cells were treated with 1% Phalloidine-FITC (Sigma Chemical Corp., USA) for 20 min and washed 3×5 min with PBS, sealed with 50% glycerin. Finally, the cells were observed and photographed under Leica TCS-SP2 Laser Scanning Confocal Microscope. Cells were seeded on cover slips in 6-well plates and fixed by absolute alcohol for 10 min, stained with HE. Images of live cells and HE were captured. The cell proliferation was determined by MTT. 2×10^3 cells/well were plated into a 96-well