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Up-regulating ribonuclease inhibitor inhibited epithelial-to-mesenchymal transition and metastasis in murine melanoma cells

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ABSTRACT

Human ribonuclease inhibitor (RI) is a cytoplasmic acidic protein. RI is constructed almost entirely of leucine rich repeats, which might be involved in unknown biological effects except inhibiting RNase A and angiogenin activities. We previously reported that up-regulating RI inhibited the growth and metastasis of melanoma cells. Epithelial–mesenchymal transition (EMT) is a critical event of cancer cells that triggers invasion and metastasis. However, the role of RI in the EMT process remains unknown. Here we hypothesize that RI might inhibit melanoma invasion and metastasis by regulating EMT. We found that over-expression of RI induced up-regulation of E-cadherin, accompanied with decreased expressions of proteins associated with EMT such as N-cadherin, Snail, Slug, Vimentin and Twist both in vitro and in vivo. Furthermore, RI restrained matrix metalloproteinase MMP-2 and MMP-9 secretions in B16 and B16-F10 melanoma cells. In addition, we also found that up-regulation of RI inhibited cell proliferation, migration and invasion as well as changed cell morphology, adhesion and rearranged cytoskeleton in vitro. Finally, the effects of RI on phenotype and invasiveness translated into suppressing metastasis by the experimental metastasis models of melanoma with lighter lung weight, a fewer metastasis nodules and a lower incidence rate, with respect to the control groups. Taken together, our data highlight, for the first time, that RI plays a novel role in inhibiting development and progression of murine melanoma cells through regulating EMT. These results suggest that RI could be a therapeutic target protein for melanoma and may be of biological importance.

1. Introduction

Ribonuclease inhibitor (RI) is an important acidic cytoplasmic protein with many functions. RI locates on the chromosome 11p15.5, near location of protooncogene ras, the study indicated that more than one locus at 11p15 could be involved in tumorigenesis. RI may relate to cell growth and differentiation (Sabbioni et al., 1997). The earliest hypothesis was that RI was involved in the regulation of intracellular levels of RNA. The second possible role for RI is to protect cells against noncytosolic RNases that gain entry into the cytosol. Another possible function of RI could be to regulate the potential biological effect of some RNase family members, for example RI inhibits angiogenic activity through binding to angiogenin (Dickson et al., 2009; Haigis et al., 2003). RI contains a large number of 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 (Sharpiro, 2001). These LRRs are present in a large family of proteins that are distinguished by their display of vast surface areas to foster protein–protein interactions. The unique structure and function of RI have resulted in its emergence as the central protein in the study of LRRs (Kobe and Kajava, 2001; Nekrasov and Zinchenko, 2010). Therefore, we presume that RI could also possess unknown biological functions. The experiments showed that RI might effectively inhibit tumor-induced angiogenesis (Botella-Estrada et al., 2001; Fu et al., 2005). We previously reported that up-regulating RI could decrease cell migration, regulate cell cycle, and induce cell apoptosis in B16 melanoma cells, as well as suppress melanoma growth and metastasis in vivo (Chen et al., 2005, 2004). Recently, we found that down-regulating RI could significantly promote growth and metastasis potential of non-invasive BIU-87 cells in vitro and in vivo (Chen et al., 2011).

The initiation of metastasis is characterized by the increased motility and invasiveness of cancer cells. To acquire such invasive abilities, tumor cells undergo physiological changes such as epithelial-to-mesenchymal transition (EMT). EMT is a process, in which cells lose generally immotile epithelial characteristics and

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gain motile mesenchymal properties (Jang et al., 2011; Lander et al., 2011). EMT is essential for normal developmental processes, including gastrulation and neural crest formation. In addition, EMT has gained attention as a critical phenotypic alteration of cancer cells to acquire invasive and metastatic abilities. EMT is mediated through several transcription repressors, such as Snail, Slug, Twist and ZEB1, mesenchymal markers Vimentin and N-cadherin, 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 (Wu et al., 2011; Palena et al., 2011).

However, the underlying mechanism by which RI inhibits metastasis has not been fully understood. Therefore, it will be valuable to examine whether RI would be a novel regulator of EMT. In this study, we hypothesize that RI regulates invasion and metastasis through EMT. The results showed that up-regulating RI inhibited murine melanoma cell proliferation and invasion, changed cell morphology and rearranged cytoskeleton. Importantly, RI transfection led to an increase in E-cadherin expression, accompanied by a decreased expression of N-cadherin and Vimentin as well as elevated production of transcription factors, such as Snail and Slug in vitro and in vivo. Intriguingly, tumor-bearing mice exhibited that RI over-expression inhibited metastasis in B16-RI and B16-F10-RI groups compared with control groups, providing important novel insights into the regulating role of RI in murine melanoma metastasis by EMT. RI could be a therapeutic target for metastatic melanoma.

2. Materials and methods

2.1. Cell lines, animal and reagents

B16 murine melanoma cells were from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, PR China); B16-F10 murine melanoma cells were obtained from the American Type Tissue Collection. Fetal calf serum was from Hyclone (Logan, UT, USA). RPMI 1640 medium and G418 were products of Gibco-BRL (Carlsbad, CA, USA). C57BL/6 mice were purchased from Experimental Animal Center of National Bio-industry Base in Chongqing. Mice were maintained according to National Institutes of Health standards for the care and use of experimental animals. Lipofectamine 2000 reagent and Trizol were bought from Invitrogen, Inc. (Carlsbad, CA, USA). Polyclonal rabbit anti-human RI antibody was prepared by our laboratory. Rabbit anti-mouse β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal rabbit anti-mouse antibodies of MMP-2, MMP-9, nm23-H1, E-cadherin, N-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 RI eukaryotic expression plasmids

Human RI cDNA sequence (accession number: NM_002939) was provided by the GenBank.

Total RNA was extracted from human QGY-7703 cells by Trizol reagent, the cDNA sequence of RI was amplified by reverse transcription-polymerase chain reaction (RT-PCR). The primers were designed as follows: forward, 5′-CGGAATTCCTTCACCTCCACCATGAGC-3′; and reverse, 5′-GCGTCGACAGGAAGACCTCAGGAGATG-3′. Annealed double-stranded oligonucleotides were inserted into the eukaryotic expression plasmid pIRES2-EGFP with DNA recombinant techniques. The recombinant plasmids were identified by endonuclease EcoRI and SalI digesting. Finally they were further verified by DNA sequencing.

2.3. Cell culture and gene transfection

The B16 and B16-F10 cells were grown in RPMI 1640 medium with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were seeded into six well plates, allowed to grow until 80–90% of the confluence, then were transfected with the plasmids using Lipofectamine 2000 reagent according to the manufacturer's instructions. Forty-eight hours after transfection, the selection was performed with 1000 μg/ml of G418 for 2 weeks and 500 μg/ml of G418 for additional 2 weeks. The individual G418-resistant monoclines were obtained by limiting dilution, then proliferated and expanded to generate stable transfected cell lines that express RI and the control blank vector, and named B16-RI or B16-F10-RI cell lines and B16 vector or B16-F10 vector cell lines, respectively. In all procedures, G418 selection was continued.

2.4. RT-PCR analysis

The total cellular RNA was isolated from the B16 and B16-F10 cells using the Trizol reagent according to manufacturer's instruction. Reverse transcription was performed and cDNAs were amplified with the following primer pairs: RI forward: 5′-TCA GCG ACA ACC TCT TGG G-3′ and reverse: 5′-CACAATGCCGCACAGGTC-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 30 s and a total 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 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.

2.5. Western blot assay

Cell total proteins were extracted using cell lysis buffer, total protein concentration was measured using Enhanced BCA Protein Assay Kit. Equal amounts (30 μg) of protein were loaded into each lane. The samples were separated by the electrophoresis in a 12% SDS-PAGE gel, and then were electrotransferred to a PVDF membrane at 200 mA for 1 h. Next, the samples were blocked with 5% skimmed milk powder in TBST buffer (0.1% Tween 20, 150 mM NaCl, and 10 mmol/l Tris–HCl, pH 7.6) overnight at 4 °C. The membrane was probed with rabbit anti-human RI antibody (1:300 dilution), rabbit anti-mouse antibodies against MMP-2 (1:750 dilution), MMP-9 (1:750 dilution), nm23-H1 (1:300 dilution), E-cadherin (1:750 dilution), N-cadherin (1:750 dilution), Snail (1:500 dilution), Slug (1:500 dilution), Vimentin (1:500 dilution), Twist (1:500 dilution) and rabbit anti-actin primary antibody (1:1000 dilution, actin of the same sample was used as an internal control) respectively for 2 h at 37 °C, washed thoroughly 3× for 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× for 10 min with TBST. 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 detection

The B16 and B16-F10 cells were incubated for 24 h on cover slips in 6-well plates and washed with PBS, fixed with 4% paraform for 30 min. The cover slips were incubated with 3% BSA in PBS