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Research paper

# Ribonuclease inhibitor up-regulation inhibits the growth and induces apoptosis in murine melanoma cells through repression of angiogenin and ILK/PI3K/AKT signaling pathway

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ABSTRACT

Human ribonuclease inhibitor (RI), a cytoplasmic protein, is constructed almost entirely of leucine rich repeats. RI could suppress activities of ribonuclease and angiogenin (ANG) through closely combining with them. ANG is a potent inducer of blood vessel growth and has been implicated in the establishment, growth, and metastasis of tumors. ILK/PI3K/AKT signaling pathway also plays important roles in cell growth, cell-cycle progression, tumor angiogenesis, and cell apoptosis. Our previous experiments demonstrated that RI might effectively inhibit some tumor growth and metastasis. Our recent study showed that ILK siRNA inhibited the growth and induced apoptosis in bladder cancer cells as well as increased RI expression, which suggest a correlation between RI and ILK. However, the exact molecular mechanism of RI in anti-tumor and in the cross-talk of ANG and ILK signaling pathway remains largely unknown. Here we investigated the effects of up-regulating RI on the growth and apoptosis in murine melanoma cells through angiogenin and ILK/PI3K/AKT signaling pathway. We demonstrated that up-regulating RI obviously decreased ANG expression and activity. We also discovered that RI over-expression could remarkably inhibit cell proliferation, regulate cell cycle and induce apoptosis. Furthermore, up-regulation of RI inhibited phosphorylation of ILK downstream signaling targets protein kinase B/Akt, glycogen synthase kinase 3-beta (GSK-3β), and reduced β-catenin expression in vivo and vitro. More importantly, RI significant inhibited the tumor growth and angiogenesis of tumor bearing C57BL/6 mice. In conclusion, our findings, for the first time, suggest that angiogenin and ILK signaling pathway plays a pivotal role in mediating the inhibitory effects of RI on melanoma cells growth. This study identifies that RI may be a useful molecular target for melanoma therapy.



## 1. Introduction

Metastatic melanoma is a life-threatening disease and no effective treatment is currently available. The incidence of melanoma is increasing worldwide, and the prognosis for patients with high-risk or advanced metastatic melanoma remains poor despite advances in the field. Therefore, it is important to understand the molecular mechanism of melanoma progression in order to search for new targets to treat advanced disease more efficiently [1].

The biological role of RI is not known in its entirety. The RI recognizes and inhibits ribonucleases by affinity. In addition, the complex of RI with Angiogenin (ANG), which stimulates neovascularization by activating transcription in the nucleus, is the tightest of known RI·ribonuclease complexes in vitro. Angiogenin is a 14.2 kDa polypeptide member of the RNase A superfamily and is the only human angiogenic factor that possesses ribonucleolytic activity [2]. Angiogenesis is a key event in tumor development and progression. Kimberly et al. report RI affects ANG-induced neovascularization of rabbit corneas, which provide the first direct evidence that RI serves to regulate the biological activity of ANG in vivo [3]. Furthermore, some reports have suggested that ANG is important in regulating rRNA transcription in cells. ANG has been shown to undergo nuclear translocation in both cancer cells and endothelial cells where it stimulates rRNA transcription, a rate-limiting step in protein translation and cell proliferation. It has been therefore proposed that ANG stimulated rRNA transcription is a general requirement for cell proliferation and angiogenesis [4–6].

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The dual role of ANG in cancer progression suggests that ANG is a molecular target for the development of cancer drugs. ANG inhibitors would combine the benefits of both antiangiogenesis and chemotherapy because both angiogenesis and cancer cell proliferation are targeted. ANG antagonists would also be more effective as angiogenesis inhibitors than others that target only one angiogenic factor [7]. Antagonists to angiogenin have been shown to inhibit the growth and angiogenesis of human tumor cells in athymic mice [8].

The experiments showed that RI might effectively inhibit tumor-induced angiogenesis [9,10].

Yet, a role for RI in angiogenesis is not fully clear in vivo [3,11]. More intriguingly, 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 [12]. These leucine-rich repeats (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 [13,14]. Therefore, we presume that RI could also possess unknown biological functions. Recently, we found that down-regulating RI could significantly promote growth and metastasis potential of non-invasive BIU-87 cells in vitro and vivo [15]. The mechanism by which RI inhibits tumor growth has not been well understood.

ILK/PI3K/AKT signaling pathway regulates multiple biological processes such as apoptosis, metabolism, cell proliferation and cell growth. Integrin-linked kinase (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 such as motility and contractility, survival, invasion, proliferation, and angiogenesis [16,17]. We lately reported that ILK siRNA inhibited the growth and induced apoptosis of human bladder cancer cells; meanwhile, experiment showed that RI and ILK have a colocalization and negative expression correlation in cytoplasm, which suggests that RI and ILK might have certain interaction or relevant function [18]. Therefore, further research on the relationship between RI and ILK is required.

Trouillon et al. reported that ANG activates nitric oxide synthase (NOS) by interacting with the cell nucleus. Similarly, NOS activity was stopped by blocking the PI3k/Akt kinase signaling transduction cascade, showing the importance of this pathway and ANG for NOS activity [19]. Kim et al. demonstrated that ANG induces transient phosphorylation of protein kinase B/Akt in cultured human umbilical vein endothelial (HUVE) cells. ANG has been shown to activate Akt. The results suggest that cross-talk between ANG and protein kinase B/Akt signaling pathways could be essential for ANG -induced angiogenesis in vitro and in vivo [20,21]. Consequently, we reason that RI and ILK/PI3K/AKT signaling pathways could realize interactive response (cross-talk) to form the regulating signal network by Angiogenin. At present there are not any reports that RI involve in regulating signal transduction pathways in order to executive its biological function. Here, we report that RI inhibits murine melanoma cell growth through suppression of angiogenin and ILK/PI3K/AKT signaling pathway. To our knowledge, this is the first demonstration of the link between RI and ILK/PI3K/AKT signaling pathway via angiogenin in 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, P.R.China); B16-F10 murine melanoma cells were obtained from the American Type Tissue Collection. Fetal calf serum was from Hyclone (Logan, Utah, 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. C57BL/6 mice were bred and held in the Experimental Animal Center of Chongqing Medical University. The protocol was approved by **The Ethics Committee of Chongqing Medical University**. Mice were maintained according to National Institutes of Health standards for the care and use of experimental animals. The care of laboratory animal and the animal experimental operation also conformed to Chongqing Administration Rule of Laboratory Animal. This housing facility is an ordinary housing facility, and it has in keeping with national standard (Laboratory Animal—Requirements of Environment and Housing Facilities, GB 14925—2001). The animal production license and animal license of Chongqing Medical University are SYXK (Chongqing)2007-0001, SCXK(Chongqing)2007-0001 and the applied animal certificate number of Xiangyang Pan is CQMULA[2011]52. Lipofectamine 2000 reagent and Trizol were bought from Invitrogen, Inc. (Carlsbad, California, USA). Polyclonal rabbit anti-human RI antibody was prepared by our laboratory, rabbit anti-mouse β-actin, ILK and CD31 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal rabbit anti-mouse antibodies of anti-mouse Angiogenin, Bcl-2, Bax, Caspase 3, Akt, GSK3(α/β), p-Akt, p-GSK3β and β-catenin were purchased from Bioworld Technology, Inc(St. Louis, USA). The rest of the primary antibodies are from Beijing Zhongshang Biotechnology (Beijing, P.R. China).

### 2.2. Construction of the plasmids

Human RI cDNA sequence (accession number: NM_002939) and human ANG cDNA (accession number: NM_001097577) were provided by the GenBank. The pIRSE2-EGFP-RI, pcDNA3.1(−)-myc-RI, The pEGFP-C1-RI and pCMV-3xflag-ANG expression plasmids were generated using standard recombination DNA technique. 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 for pIRES2-EGFP plasmid (Clontech)were designed as follows: forward, 5′-CGGAATTCCTTCACCTCCACCATGAGC-3′, and reverse, 5′-GCGTCGACAGGAAGACCTCAGGAGATG-3′; the primers for pEGFP-C1 plasmid (Clontech) is following: forward, 5′-CGGAATTCCACCATGAGCCTGGACATCC-3′, and reverse, 5′- GCGTCGACAGGAAGACCTCAGGAGATG -3′; and the pcDNA3.1(−)-myc vector (Invitrogen) was used the following primer pair: 5′-GCTCTAGAATGAGCCTGGACATCAGAG- 3′, 5′-CCCAAGCTTGGAGATGACCCTCAGGGAT-3′, Annealed double-stranded oligonucleotides were inserted into the eukaryotic expression plasmids with DNA recombinant techniques. The recombinant plasmids pIRSE2-EGFP-RI and pEGFP-C1-RI were identified by endonuclease EcoR I and Sal I (underlined) digesting, and the pcDNA3.1(−)-myc-RI was identified by endonuclease Xba I and HindIII (underlined) digesting. Finally they were further verified by DNA sequencing. The human ANG, including a open reading frame of 444 bp, pCMV-3xflag-ANG was generated by cloning the ANG sequence into the HindIII and BamHI (underlined) site of the pCMV-3xflag vector (Promega) using the following primer pair: 5′-CCCAAGCTTATGGTGATGGGCCTGGGCG-3′, 5′-CGGGATCCGCTGGTTACGGACGACGG-3′. All plasmids were verified by nucleic acid sequencing; subsequent analysis was performed using BLAST software (available on the World Wide Web at ncbi.nlm.nih.gov/blast/).