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Loss of Raf kinase inhibitor protein is associated with malignant progression in hepatic fibrosis

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ABSTRACT

Raf kinase inhibitory protein (RKIP), besides regulating important intracellular signaling cascades, was described to be associated with progression, metastasis and prognosis in several human neoplasms. But its role in hepatic fibrogenesis remains unclear. In the present study, we found that the absence of RKIP expression significantly enhanced the proliferation of HSC-T6 cells. Reduced RKIP expression promoted the activation of HSCs and the accumulation of collagen, as evidenced by the increases in the levels of collagen I and α-smooth muscle actin. Moreover, down-regulating RKIP expression led to severe histopathological changes and collagen accumulation in hepatic tissues of rats with liver fibrosis. Furthermore, the absence of RKIP promoted the activation of ERK/MAPK pathway *in vitro* and *in vivo*. Our findings clearly demonstrate an inverse correlation between RKIP level and the degree of the liver injury and fibrosis. Loss of RKIP may be associated with malignant progression in hepatic fibrosis.

1. Introduction

Liver fibrosis is characterized by the accumulation of extracellular matrix (ECM) proteins like collagen [1]. It is reversible wound-healing response against a variety of acute and chronic stimulation in liver, including ethanol, viral infection, drugs and toxins, cholestasis and metabolic disease, all of which would disturb the balance between repair and scar formation in the organ. Previous studies showed that the proliferation and activation of hepatic stellate cells (HSCs) played a key role in the development of liver fibrosis [2]. Targeting the proliferation of HSCs has been considered as a novel therapeutic strategy for treating liver fibrosis [3,4].

It has been reported that the extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway is involved in cell proliferation, differentiation and migration. The ERK signal pathway has been found to play an important role in hepatic fibrosis *via* regulating the ECM synthesis in activated HSCs [5]. Recently, the Raf kinase inhibitor protein (RKIP) was identified as an inhibitor of the ERK/MAPK signaling pathway, it is a conserved cytosolic protein with wide tissue expression and does not share significant homology with other kinase inhibitors [6]. Many studies showed that RKIP directly interacted with both Raf-1 and MEK and disrupted the Raf-1/MEK interaction, thereby preventing the activation of MEK and downstream components of the signaling cascade. Over-expression of RKIP suppressed MAPK signaling, and down-regulation of RKIP had the opposite effect [7]. Additionally, RKIP not only interfered with the Raf-1-MEK1/2-ERK1/2 signal transduction pathway but also inhibited the signal transduction of NF-κB and G protein coupled receptor kinases (GRKs) [8,9]. RKIP suppresses the activity of the nuclear factor-κB-Snail circuitry and inhibits the epithelial-to-mesenchymal transition program, which is a pivotal step in tumor invasion and the formation of metastasis [10].

Although the molecular mechanism by which RKIP inhibits the ERK/MAPK signaling pathway has been partially delineated, little is known about the role of RKIP in liver fibrogenesis and how RKIP may be regulated in hepatic stellate cells. In the present work, we aimed to assess, *in vitro* and *in vivo*, the biological consequences of RKIP down-regulation on aggressiveness of liver fibrosis.

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2. Materials and methods

2.1. Cell line

Rat hepatic stellate cell line (HSC-T6) were purchased from Shanghai Meixuan Biological Science and Technology Ltd. (Shanghai, China). HSC-T6 cells were maintained in Dulbecco's modified Eagle's medinm (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air–5% CO_2.

2.2. Small interfering RNA for RKIP

To inhibit RKIP expression, a specific small interfering RNA for RKIP (RKIP-siRNA: 5′-GATTCAGGGAAGCTCTACA-3′) and non-targeting control (Control-siRNA: 5′-GCGACCGACGAGTTATTAA-3′) were transferred into the pGenesil-2.1 vector (Genesil Biotechnology, Wuhan, China) according to the manufacturer's instructions. Then, the vector (pGenesil-RKIP-siRNA or pGenesil-Control-siRNA) was transfected into HSC-T6 using Lipofectamine™ 2000 (Invitrogen Corporation, CA, USA). Forty-eight hours later, the medium was renewed and the cells were rinsed with PBS thrice and extracted for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in lysis buffer.

2.3. Cell proliferation assay

After 36 h of transfection, HSC-T6 cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., USA) assay. Results were presented as the average absorbance of six wells in one experiment and the assays were performed in triplicate.

2.4. Measurement of intracellular collagen content

The collagen content was quantified by the Sirius Red-based colorimetric assay [11]. Briefly, After 36 h of transfection, HSC-T6 cells were washed with phosphate buffered saline (PBS), followed by fixation with Bouin's fluid for 1 h. The fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 h on the rocker with the speed of about 5 rpm. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and the absorbance was measured at 550 nm against 0.1 N NaOH as a blank.

2.5. RT-PCR assay

Total RNA was isolated from HSC-T6 using a TRIzol kit (Invitrogen, CA, USA). cDNA synthesis was performed using SuperScript II (Invitrogen Corp., Carlsbad, CA). Gene expression levels were measured by real-time PCR on an ABI7300 machine (Applied Biosystems, CA) with iTaq™ SYBR_ Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA, USA). The primers used in this study are listed in Table 1. Threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). The relative expression levels of the genes of interest were calculated using the DDCt method [12]. The results were normalized against GADPH and presented as target mRNA to GADPH ratio.

2.6. Hepatic fibrosis model in rats

Male SD rats (200 ± 10 g) were obtained from Guangxi Medical University Experimental Animal Centre (Nanning, Guangxi, China) and were allowed to acclimate in quarantine for a week prior to experimentation. The research was conducted according to protocols approved by Guangxi Medical University Institutional Ethical Committee. In this study, locostatin (Merck, Darmstadt, Germany), a non-antibacterial oxazolidinone derivative, was used as an specific inhibitor of RKIP [13].

The hepatic fibrosis was induced by CCl_4 in rats as previously described [14]. Briefly, following one week acclimation period, rats were randomly divided into four groups of 15 rats per group, including normal control group, locostatin control group, CCl_4 model group and locostatin-treated group. The rats in the locostatin control group were only administrated with 0.5 mg/kg locostatin intraperitoneally once a day for 12 weeks, while the animals in the normal control group were received equivalent normal saline. The rats in the model group and the locostatin-treated group received 2 ml/kg CCl_4 (mixed 1:1 in peanut oil) intragastrically twice a week for 12 weeks. In addition to CCl_4 treatment, rats in the locostatin-treated group received 0.5 mg/kg locostatin intraperitoneally once a day for 12 weeks. All rats were sacrificed at the end of treatment. Blood and liver samples were obtained for further examination.

2.7. Histological analysis of liver

Liver tissues were fixed in 10% formalin, embedded in paraffin and sectioned at 5 μm thickness. Changes in liver pathology and collagen deposition were observed by hematoxylin-eosin (H&E) staining and Masson's trichrome staining [15].

2.8. Determination of collagen-related indicators in rats

The analysis of hydroxyproline (Hyp), type III precollagen (PCIII), laminin (LN) and hyaluronic acid (HA) were used to directly determine the quantity of collagen and can be used as a measure to reflect the degree of hepatic fibrosis [16]. In the present study, the levels of Hyp, PCIII, LN and HA in liver tissues were determined by using commercially available kits (Nanjing Jiacheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions [17].

2.9. Immunohistochemistry analysis for hepatic RKIP and ERK

Representative liver tissue sections were used to immunohistochemical analysis. Briefly, fixed tissues were embedded in paraffin blocks and sectioned into 4 μm slices. Tissue sections were then deparaffinized, rehydrated in ethanol gradient and stained

Table 1
The sequences of primers used for real-time quantitative PCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)
Type 1 collagen	CAGTCGCTTCACCTACAGCA	GGTGGAGGGAGTTTACACGA
α-SMA	AGGCACCCCTGAACCCCAA	CAGCACCGCCTGGATAGCC
MMP-1	TGATATCGGGGCTTTGATGT	CACTTCTCCCCGAATCGTAG
MMP-2	TCCCATTTTGATGACGATGA	CCGTACTTGCCATCCTTCTC
GADPH	CTCAGACACCATGGGGAAGGTGA	ATGATCTTGAGGCTGTTGTCATA