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Down-Regulation of c-Met Expression Inhibits Human HCC Cells Growth and Invasion by RNA Interference

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Background. **Cell migration is a basis for invasion and metastasis of malignant tumors. Receptor tyrosine kinases are recognized as important therapeutic targets in antineoplastic strategies. C-met is a receptor tyrosine kinase highly expressed in human hepatocellular carcinoma (HCC) cell line MHCC97-H. Higher expression of c-met in tumor tissue can lead to scattering, angiogenesis, proliferation, enhanced cell motility, invasion, and eventually, metastasis. To explore the roles of c-met in modulating the motility of cell, we silenced c-met expression in the HCC line MHCC97-H by RNA interference (RNAi).**

Materials and Methods. **For transient expression, c-met-siRNA 1,2 recombinant plasmids were transfected into phoenix A cells. The MHCC97-H cells were cultured in Dulbecco's modified Eagles's medium (DMEM) with 10% fetal bovine serum (FBS) to establish MHCC97-H HCC cells stably expressing c-met-siRNA. MHCC97-H cells were treated with the recombinant virus for assay of c-met mRNA and protein, evaluation of growth and invasion of MHCC97-H cells, and identification of hepatitis B virus X (HBX) protein correlation with c-met.**

Results. **After transfection of c-met-siRNA for 48 h, the expression of c-met decreased markedly in MHCC97-H cells; the most effective site of the siRNA target sequence is at the 537 upstream, far from the transcription start. In addition, the proliferation, motility, and invasive ability of MHCC97-H cells were significantly inhibited. Furthermore, we showed that hepatitis B virus (HBV) X protein (HBX) potentiated the activities of the extracellular signal-regulated kinase 1/2 (ERK1/2) in MHCC97-H cells. Treatment with extracellular signal-regulated kinase (ERK) inhibitor (U0126), but not P38 MAPK inhibitor (SB203580) or phosphatidylinositol 3-kinase (PI3K) inhibitor (wortmannin), markedly suppressed the expression of c-met protein in MHCC97-H cells.**

Conclusion. **These results indicate that the overexpression of c-met protein plays an important role in the cell invasion of MHCC97-H, and HBX protein may promote the expression of c-met by ERKs pathway.** © 2010 Published by Elsevier Inc.

Key Words: **c-met; siRNA; hepatocellular carcinoma; invasion; hepatitis B virus X protein; extracellular signal-regulated kinase 1/2.**

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors that rates fifth in incidence and third in mortality worldwide [1]. Despite improvement in surveillance and clinical treatment, there is still no satisfactory method to increase the survival rate of patients, especially in those who accompanied with intrahepatic and distant metastasis. For this reason, understanding the mechanisms underlying HCC as well as identifications of new molecular targets are of great importance. Hepatocellular growth factor (HGF) and its receptor (c-Met) pathway have been strongly implicated in the promotion of the invasive/metastatic tumor phenotype, such as lung cancer invasion and metastasis. Studies have shown that activation of HGF/c-met signaling could increase the motility of epithelial cells, Madin-Darby canine kidney cells [2], and the induction of invasiveness in Caco-2 colon cancer epithelial cells with poor aggression [3].

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HCC cell invasion and metastasis are multi-stage processes involving various classes of proteins, including cell-adhesion molecules, extracellular proteases, angiogenetic factors, and growth factor. Among them, HGF/c-met have been strongly implicated in the metastasis of HCC [4–6]. The prognosis for HCC largely depends on the clinicopathologic characteristics regarding invasion and metastasis; c-met protein was shown to be highly expressed in multiple tumors, including the gastrointestinal tract, thyroid, metastatic melanoma, prostatic, and breast carcinoma [7–11]. It has been reported that MHCC97-H human HCC line, constitutively overexpressing c-met, has invasion capability [12]. In addition, our recent studies also shown c-met is higher c-met expression in human HCC tumor than in normal liver tissues by immunohistochemical staining, and its expression is linked to some known clinicopathologic parameters, such as lymph node status, capsule, metastasis (intra- or extrahepatic metastasis), and histological grade. Overexpression of c-met is associated with abnormal E-cadherin, β-catenin expression, and tumor invasive potential, suggesting that it may serve as a prognostic marker in HCC [13].

This study aims to further clarify effect of overexpression of c-met on invasion and metastasis in hepatoma cell lines. A siRNA specifically suppressing expression of c-met was stably transfected into HCC cell line. Results showed that down-regulation of c-met significantly inhibits the growth and invasion of human HCC cell *in vitro*. Furthermore, hepatitis B virus X (HBX) protein may promote the expression of c-met through ERKs pathway.

MATERIALS AND METHODS

Construction of Recombinant pSuppressor Retro/met-siRNA

Eight RNAi sequences targeting c-met were designed *via* the siRNA online program (www.ambion.com). Then the two targeting sequences, c-met-siRNA-1 and c-met-siRNA-2, were applied following initial screening. Three pairs of complementary c-met siRNA oligonucleotide single chains (including a random control chain for siRNA specialty) were synthesized by Sangon Biological Engineering (Shanghai, China). These sequences were shown in Fig. 1. Single-strand DNA were diluted in 100 μL TE. PCR program was set up as follows: 94°C 2 min → 68°C 10 min → 37°C 10 min → 4°C. The pSuppressorRetro vector was double digested with SalI and XbaI to generate compatible ends to make recombinant plasmid *via* standard protocols. Inserts in recombinant plasmids were all confirmed by DNA sequencing.

Transfection of siRNA Expression Plasmid

For transient expression, Phoenix A cells (1×10^6) were seeded in 6-well plates and cultured overnight at 37°C in a 5% CO_2 incubator. The next day, siRNA recombinant plasmids were transfected into Phoenix A cells using lipofectamine according to he manufacturer's instructions (Invitrogen, Carlsbad, CA). After 48 h incubation at 37°C in a 5% CO_2 incubator, the supernatant containing viral particles were gathered to infect target cells immediately or stored at –80°C.

Establishment of MHCC97-H HCC Cells Stably Expressing c-met-siRNA

The MHCC97-H cells (purchased from Institute of Liver Cancer, Zhongsan Hospital affiliated to Fudan University, Shanghai, China) were cultured in Dulbecco's modified Eagles's medium (DMEM) with 10% fetal bovine serum (FBS), 100 μg/l mL penicillin, and 100 μg/mL streptomycin; 2×10^5 cells/mL MHCC97-H cells were seeded on the 60 mm dishes, and the medium was changed to 3 mL of fresh after 24 h. The supernatant containing viral particles produced by Phoenix A was added. The Polybrene (Sigma -Aldrich, St. Louis, MO) was added to this medium to a final concentration of 8 μg/mL with gentle shakng. Every 24 h, the fresh viral supernatant was added up to three times in all. After 48 hs post-infection, the medium was discarded, and the cells were seeded in the selective medium containing G418 (400 μg/mL). The selective medium was changed every 2 to 3 d. Colonies ere formed about 7 to 10 d later. Then the cells cloned were pick up and continued for culture until confluence, and collected to be stored for further use.

Reverse Transcription PCR, Real-Time Fluorescent Quantitative PCR Assay

Infected MHCC97-H cells were harvested and washed with phosphate buffered saline (PBS). Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction. Cells were lysed directly in the flasks, and RNA samples were stored in –70 °C for further use. One μg of each RNA was transcribed to cDNA using RT-PCR kit (Takara, Kyoto, Japan). cDNA was stored at –20°C. The primer sequences used in polymerase chain reaction (PCR) were as follows:

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward primer):5′-CACCAGGGCTGCTTTTAACTC-3′

GAPDH (reverse primer):5′-GATGATGACCCTTTTGGCTCC-3′

c-met (forward primer):5′-GAGGTTCACTGCATATTCTCC-3′

c-met (reverse primer):5′-CACTGATATCGAATGCAATGG-3

Transcripts were quantified by real-time fluorescent quantitative (FQ-PCR) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan).

Western Blot Analysis

Infected MHCC97-H cells were harvested in lysis buffer, and an equal amount of cellular protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and blots were probed with rabbit anti-human c-met antibody (Santa Biotechnology, Santa Cruz, CA) and rabbit anti-human HBX antibody (Sigma-Aldrich, St. Louis, MO) (1:500). Blots were washed 3×5 min with Tris-buffered saline/0.1% Tween 20 and incubated with sheep anti-rabbit IgG-HRP antibody (Zhongshan company Co., Beijing, China) for 1 h. The membranes were washed again as described above, and the bands were detected by chemiluminescence for visualization. β-Actin protein was used as an internal control. The expression level of c-met was presented by the optical density (OD) ratio of c-met/β-actin. To further determine which signaling pathway is involved in the activation of c-met expression, we also detected the expression of HBX, p-ERK, and ERK (Santa Cruz Biotechnology), and c-met protein. Cells were incubated for 24 h in serum-free DMEM with or without U0126 (5 μM), SB203580 (SB) (5 μM), or wortmannin (Wort) (5 μM). MHCC97-H cell lysates were size-fractionated by SDS- PAGE and transferred to PVDF membranes and then analyzed by Western blot using HBX, c-met, p-ERK, and ERK antibodies.