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Original Articles

Hepatocyte growth factor (HGF) upregulates heparanase expression via the PI3K/Akt/NF-κB signaling pathway for gastric cancer metastasis

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ABSTRACT

Heparanase (HPA) is an endoglucuronidase that can promote the shedding of associated cytokines in several types of tumors. However, little is known about what controls the expression of HPA or its role in gastric cancer. In this study, we report for the first time that HGF regulates HPA expression to promote gastric cancer metastasis. In this study, HGF and HPA were found to be significantly expressed in 58 gastric cancer patients. High expression of both HGF and HPA was positively associated with TNM stage, invasion depth and poor prognosis. In MKN74 cells, exogenous HGF significantly increased HPA expression at both the mRNA and protein levels. Further study revealed that HGF first activated PI3K/Akt signaling. NF-κB signaling was activated downstream of PI3K/Akt and promoted HPA expression. However, when c-met, PI3K/Akt or NF-κB signal inhibitors were used, HPA expression was significantly decreased. All of these results indicate that HGF regulates HPA expression by PI3K/Akt and downstream NF-κB signaling. Using bioinformatics and the ChIP assay, p65 was observed to bind to the HPA promoter. Furthermore, HGF significantly induced tumor cell migration, whereas treatment with an NF-κB inhibitor decreased migration. Moreover, when HPA was overexpressed in MKN74 cells, migration was significantly enhanced, and the HGF concentration was increased. However, when HPA was down-regulated in MKN45 cells, migration and HGF levels decreased. Together, these results demonstrate that HGF/c-met can activate PI3K/Akt and downstream NF-κB signaling to promote HPA expression and subsequent tumor metastasis.

Introduction

Gastric cancer is one of the most common malignancies and is a leading cause of cancer death worldwide, especially in Eastern Asia. A recent study reported that 640,000 men and 350,000 women are diagnosed with gastric cancer, and 464,000 men and 273,000 women die from gastric cancer every year [1]. Metastasis is the cause of 90% of all deaths from cancer and exhibits a unique set of clinical characteristics [2]. At present, tumor cell metastasis was believed to require the acquisition of certain phenotypes. First, metastatic cancer cells must exhibit migratory and invasive capacities, which are acquired through diverse alterations in gene expression and epithelial-mesenchymal transition (EMT) [3,4]. During this process, matrix metalloproteinases (MMPs) play important roles because they can degrade essentially all extracellular matrix (ECM) components [5].

In addition to MMPs, heparanase (HPA) also plays important roles in tumor metastasis. HPA is an endoglucuronidase that cleaves heparan sulfate chains of proteoglycans (HSPG) [6–8]. Heparan sulfate proteoglycans are key components of the ECM that regulate several aspects of cancer biology, including angiogenesis, tumor growth, and metastasis [9,10]. The cleavage of heparan sulfate proteoglycans can promote the shedding of heparin-binding growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), from the ECM to enhance endothelial cell migration and proliferation [11–13]. Few studies have examined the regulation of HPA expression. For example, epigenetic changes and/or mutational inactivation of p53 during cancer development may provide a molecular explanation for the induction of HPA expression observed in many human tumors [14]. Other studies have also reported that early growth response 1, SP1 and Ets transcription factor families are associated with the expression of HPA [11,15–19].

Abbreviations: HGF, hepatocyte growth factor; HPA, heparanase; HSPG, heparan sulfate chains of proteoglycans; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; MMPs, matrix metalloproteinases; ECM, extracellular matrix; ChIP, chromatin immunoprecipitation; p-AKT, phospho-AKT; p-p65, phospho-p65.

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Furthermore, our previous studies also demonstrated that multiple antigenic peptide vaccines based on cytotoxic T lymphocyte epitopes of human heparanase can be used as potent immunogens for tumor immunotherapy, which significantly decreased tumor growth and metastasis [20–22]. So it was very significant to explore the expression of HPA in cancer tissues.

HGF, through interactions with the special receptor c-met, is believed to play important roles in many tumors, including breast cancer, hepatocellular carcinoma, osteosarcoma and brain tumors [23–26]. HGF/c-met can activate a series of signaling pathways, including PI3K/Akt, ERK, NF-κB, and STAT3 [26–28]. However, whether these signaling pathways can induce the expression of HPA remains unknown. In this study, we present the first report, to our knowledge, that HGF/c-met activates PI3K/Akt signaling, followed by downstream activation of NF-κB signaling and p65 binding to the promoter of HPA to promote HPA expression and gastric cancer metastasis.

Materials and methods

Tissue specimens

Human gastric tumor specimens and corresponding para-cancer normal specimens were randomly obtained from 58 patients with gastric cancer at Southwest Hospital, Chongqing, China (during 2010 and 2011). All patients were diagnosed pathologically according to the American Joint Committee on Cancer (AJCC) criteria. The following data were recorded: age, sex, differentiation, metastasis, TNM stages, tumor size, lymph node metastasis and depth of invasion. Acquisition of tissue specimens and the study protocol were approved by the Institutional Review Board of the Third Military Medical University.

Immunohistochemistry

Standard ABC peroxidase techniques were used for immunohistochemistry (IHC). First, the slides were incubated with an anti-HGF (Santa Cruz Biotechnology, CA, USA) or anti-HPA antibody (Santa Cruz Biotechnology, CA, USA). Labeling was detected by adding biotinylated secondary antibodies (Maxim-Bio, Fuzhou, China) and avidin–biotin complex (Maxim-Bio), and slides were stained with DAB (Maxim-Bio). Finally, the slides were counterstained with Mayer's hematoxylin.

Cell culture

The gastric cancer cells lines AGS, SGC-7901, MKN45, MKN74 and BGC-832 were obtained from the American Type Culture Collection. The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Antibodies, reagents and plasmids

The following primary antibodies were used: anti–c-Met, anti-HGF, anti-Heparanase, anti-AKT, anti-phospho-AKT (Ser473), anti-p65, anti-phospho-p65 (Ser536), anti-GAPDH, anti-mouse secondary antibody (Fisher Scientific, Pittsburgh, PA) and anti-rabbit secondary antibody. The following reagents were also used: recombinant human HGF (Chemicon, Billerica, MA), c-met inhibitor, SU11274, PI3K/Akt inhibitor, GSK 690693 and NF-KB inhibitor, and Bay 11–7082 (Santa Cruz Biotechnology, CA, USA). As previously described, the HPA-cDNA plasmid was a gift from Drs. Parish and Hulett from Australia [29]. The plasmid was used to the expression of HPA in MKN74 cells relative to the NC-vector plasmid. Sh-RNA-HPA and sh-RNA-NC plasmids were purchased from the Genepharma Biotechnology Corporation (Shanghai, China) and were used to interfere with the expression of HPA in MKN45 cells. The HPA promoter was constructed by Genemine Biotechnology Corporation (Chongqing, China). Its identity was confirmed by gene sequencing of –1721–+106 bps, and the promoter was used for Luciferase Assay in MKN74 cells.

Quantitative real-time PCR (qPCR)

Total RNA from MKN74 and MKN45 cells was isolated using TRIzol reagent (Invitrogen). cDNA was synthesized using the Standard Two-step RT-PCR kit from 1 μg of total RNA in a final volume of 100 μl. Quantitative RT-PCR reactions were performed with FastStart SYBR Green Master mix (Roche) and MyIQiCycler (Bio-Rad). Fluorescence intensity was measured at the end of each elongation step as a means to evaluate the amount of formed PCR product. GAPDH was used as a reference to normalize the samples. The primers were as follow: GAPDH: forward: 5-CACCCACTCCTCCACCTTTGAC-3, Reverse: 5-GCAACTGTGAGGAGGGGAGATT-3; HPA: Forward: 5-CCCTCGTTCCTGTCCGTCAC-3′, Reverse: 5-GGGCCATTCCAACCGTAACTTC-3′.

Western blotting

Cell lysates were separated by SDS/PAGE on 10% tris–glycine gels and transferred to an NC membrane. Proteins were detected using primary antibodies at 4 °C overnight. Protein bands were visualized using enhanced chemiluminescence reagent (Amersham) on Kodak film. Equal protein loading was ensured by normalizing to the house-keeping protein GAPDH.

Cell proliferation assay

MKN74 and MKN45 cells were seeded into 96-well plate for each well (5000 cells) and cultured overnight. Then the cells were treated by PBS or HGF for 24h. Cell viability was evaluated with Cell Counting Kit-8 (CCK8; Dojindo, Japan) according to the manufacturer's protocol. After being treated with CCK8 at 37 °C for 1 h, cells were used to measure the absorbency at 450 nm using a microplate reader.

Scratch wound assay

Confluent gastric cancer cells were serum-deprived for 48 h, and wounds were generated using a sterile 200 μl pipette tip. Cells were then exposed to specified treatments (HGF, SU11274 or DMSO followed by HGF) and grown for an additional 24 h. Wound closure was assessed using an Olympus fluorescent microscope. The widths of the gaps were measured by Image-Pro Plus software, and the data acquired from 4 areas of the wound on each plate were averaged to obtain the mean gap width at a given time.

Migration assay

Gastric cancer cell lines (5×10^4 cells) were seeded into the upper compartment of Falcon™ migration inserts (8-lm pore size) with 200 μl serum-free medium. Specified treatments were added to the lower compartment of serum-free medium. After growth for 24 h, the incubation medium plus cells were removed from the top chamber using cotton swabs and PBS. The number of cells that invaded the underside of the membrane was determined. Membranes were fixed with absolute ethanol and stained with toluidine blue. Cells were counted in a double-blind manner in five consecutive fields each with a light microscope.

Chromatin immunoprecipitation (ChIP) assay

MKN74 cells were treated with PBS or HGF for 24 h. ChIP assay was performed following the manufacturer's protocol (Millipore) as previously described [30]. Approximately 5 μg anti-p65 and rabbit anti-IgG antibodies were used for immunoprecipitation, and immunoprecipitate complexes were collected with Dynal magnetic beads (Invitrogen). The primers used in the ChIP assay were as follow: BS1: Forward: 5-AATGTTGAGCAACATCACAATAC-3; Reverse: 5-GTGTCAGAATCGGGATGTGAAGTGTC-3; BS2: Forward: 5-GTTGATAAAGAATTTTGGGTGG-3, Reverse: 5-GCGTTTCCGACTCCCAGATCCC-3; BS3: Forward: 5-TGGGTTCCCACGAGAGCGCGCAG-3, Reverse: 5-CTCCCACTGCTCCCAATCCAACC-3.

Table 1
Relationship between HGF, HPA expression and clinicopathological features of gastric cancer patients.

Variable	No. of cases	HGF		P	HPA		P
		Low	High		Low	High	
Gender							
Female	21	4	17	0.96	5	16	0.62
Male	37	9	28		11	26	
Age (y)							
≥55	30	7	23	0.81	11	19	0.78
<55	28	4	24		4	24	
Tumor size							
≥5 cm	21	2	19	0.32	3	18	0.48
<5 cm	37	10	27		13	24	
Differentiation degree							
Well/moderately	18	5	13	0.71	4	14	**0.04**
Poorly	40	8	32		12	28	
Depth of invasion							
T1 + T2	32	7	25	**0.007**	10	22	**0.006**
T3 + T4	26	6	20		5	21	
Lymph node metastasis							
Yes	42	5	37	**0.01**	8	34	0.07
No	16	7	9		10	6	
TNM stage							
I + II	16	10	6	**<0.001**	9	7	**0.001**
III + IV	42	3	39		7	35	

P < 0.05 was considered it has significant difference.