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• ORIGINAL RESEARCH •

SF/HGF-c-Met autocrine and paracrine promote metastasis of hepatocellular carcinoma

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Abstract

AIM: **To explore the role of SF/HGF-Met autocrine and paracrine in metastasis of hepatocellular carcinoma (HCC).**

METHODS: **SF/HGF and c-met transcription and protein expression in HCC were examined by RT-PCR and Western Blot in 4 HCC cell lines, including HepG2, Hep3B, SMMC7721 and MHCC-1, the last cell line had a higher potential of metastasis. sf/hgf cDNA was transfected by the method of Lipofectin into SMMC7721. SF/HGF and c-met antibody were used to stimulate and block SF/HGF-c-met signal transduction. Cell morphology, mobility, and proliferation were respectively compared by microscopic observation, wound healing assay and cell growth curve.**

RESULTS: **HCC malignancy appeared to be relative to its met-SF/HGF expression. In MHCC-1, c-met expression was much stronger than that in other cell lines with lower potential of metastasis and only SF/HGF autocrine existed in MHCC-1. After sf/hgf cDNA transfection or conditioned medium of MHCC-1 stimulation, SMMC7721 changed into elongated morphology, and the a bilities of proliferation ($P<0.05$) and mobility increased. Such bio-activity could be blocked by c-met antibody ($P< 0.05$).**

CONCLUSION: **The system of SF/HGF-c-met autocrine and paracrine played an important role in development and metastas is potential of HCC. Inhibition of SF/HGF-c-met signal transduction system may reduce the growth and metastasis of HCC.**

Subject Headings: hepatocyte growth factor/Scatter factor; c-met; hepatocellular carcinoma; metastasis

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INTRODUCTION

The human proto-oncogene c-met encodes a Mr. 190, 000 heterodimeric transmembrane protein with structural features of a tyrosine kinase receptor and is expressed predominantly on epithelial cells. Its ligand HGF is a mesenchymal protein which is identical to Scatter factor (SF), a factor secreted by fibroblasts. It is well known that SF/HGF has a special binding with c-met, mainly attending mitogenic, motogenic and morphogenic effects on normal targeted cells[1-4]. Recently, its signal pathway has been explored a lot, including STAT in tubulogenesis[5], Gab-1 in cell growth[6,7], MAPK in morph ogenesis[8,9], and PI-3K in cell mitogenesis[10,11]. Its relationship with ad hesive factors[12-16] and apoptotic factors[17,18] and the cross-talk between HGF/SF-c-Met and other growth factors[19] and proto-oncogenes[20] have also bee n discussed. However, in cancer research, controversial results have been found in various tumors. Some reports suggested that HGF/SF was potent stimulator for tumor proliferation and motility[21-29], some reports made exactly opposite conclusions[30-33]. Among all the tissues, situation in liver becomes the most complicated, since SF/HGF attends all the stages of liver growth, regeneration, cirrhosis and carcinoma. SF/HGF was first found as a serum derived factor that stimulated proliferation of primary liver cells, acted as one of the initial factors in liver regeneration[34-35] and reversed cirrhosis by suppressing the increased TGF betal, fibrogenesis and hepatocyte apoptosis[36]. It ha s been unexpectedly reported to inhibit the growth of hepatoma cell in many reports[31]. Little has been known about the mechanism.

We studied a HGF/SF autocrine hepatocellular carcinoma (HCC) cell line which has a high potential of metastasis and compared its characteristic with several other HCC cell lines which do not have these features. Gene transfection and other interfering methods were used to further demonstrate the SF/HGF autocrine and paracrine on malignant capability of HCC.

MATERIALS AND METHODS

Cells

SMMC7721, HepG2, and Hep3B human HCC cell lines were purchased from Shanghai Institute of Cell Biology, Chinese Acadamy of Science (Shanghai, China). MHCC-1 was a cell line with high potential of metastasis from a resected lesion of a HCC patient. Cells were cultured in DMEM supplemented with 100 $mL \cdot L^{-1}$ fetal calf serum or AB serum (MHCC-1), penicillin (100 $kU \cdot L^{-1}$), streptomycin (100 $kU \cdot L^{-1}$).

Collection of MHCC-1 conditioned medium (MHCC-1-CM)

Cells were planted in 100 ml culture bottles. When 80% cells were subconfluent, they were washed and replaced with serum free DMEM (0.1 $mL \cdot cm^{-2}$). Three to five days later, conditioned medium was collected, centrifuged under 2000 $r \cdot min^{-1}$ for 20 minutes and stored at -20℃ until use.

Plasmid and DNA transfection

The plasmid pBS7.3 containing human full-length sf/hgf(kindly provided by Dr. George Vande Woode) was cloned into the BamHI-ApaI site of the p cDNA3.0(+) mammalian expression vector. SMMC7721 HCC cell line was transfected overnight with the constructed plasmid, using lipofectamine (Gibco). Cells were selected by G418. Colonies of cells were trypsinized within cloning rings, then they were transferred to 24-well dishes, and grown to confluency. After conditioned medium was changed to serum free DMEM for 3 d, both supertenant and whole lysised cells were screened for SF expression (ELISA, R&D). The highest expressing

clone (SF7721) was selected for further research.

RT-PCR

Total RNA was extracted (QIAGEN) and 1 μg was reversing transcribed in a 25 μL volume using Super rScriptII™ (Gibco), according to the manufacturer's instructions. Five μL reverse transcription product was used for amplification with the following primers: SF: 5'-CAG CGT TGG ATT CTC AGT AT-3', 5'-CCT ATG TTT GTT CGT GT T GGA-3'. c-met: 5'-ACA GTG GCA TGT CAA CAT CGC T-3', 5'-GCT CGG TAC TC T ACA GAT TC-3'. Forty cycles were performed, each consisting of 95℃, 45 s; 60℃, 1 min. There was a time delay for 7 min at 72℃.The reaction products were visualized by 15 g·L^{-1} agarose gel electrophoresis.

Western blotting

Goat anti-human HGF antibody was purchased from R&D. Rabbit anti-human c-met anti body, anti-rabbit IgG-AP and anti goat IgG-AP were purchased from Santa Crus. Cells were washed with PBS, and lysed at 0℃ for 30 min in lysis buffer (TrisCl 50 mmol·L^{-1} pH8.0, NaCl 150 mmol·L^{-1}, NaN_3 0.2 g·L^{-1}, PMSF 100 mg·L^{-1}, Aprotinin 2 g·L^{-1} , TritonX-100 10 g·L^{-1}). In c-met detection, protein content was examined using BCA™ Protein Assay (Pierce), and 20 μg protein per lane was electrophoreses on 80 g·L^{-1} SDS polyacrylamide gels after boiling for 5 min in 2X loading buffer. As to SF/HGF, conditioned medium was mixed with 2X loading buffer and added to 100 g ·L^{-1} SDS polyacrymide gels. Protein was blotted onto nitrocellulose membranes. After electrobloting, the membranes were blocked in PBS-50 mg·L^{-1} non-fat dry milk, washed with PBS-Tween buffer, and incubated with the primary antibody (1:500) diluted in blocking buffer for 2 h. Membranes were then washed, incubated with the appropriate second antibody (1:500) in blocking buffer for 2 h, and re-was hed. Blotted proteins were stained with BICP-DAB (Huamei).

Cell scatter and morphology

5×10^3 cells of SMMC7721 and SF7721 were planted into the wells of 96-wells plates. Three replicated wells. Twenty-four hours later, wells were washed with PBS and fixed with ethanol and stained with Giemsa and examined under an inverted microscope. To explore the role of SF/HGF-c-met paracrine, 5×10^3 cells of SMMC7721 were planted into the wells of 96-wells plates. And 200 μL MHCC-1-CM was added into each well within diluted range 1:2, 1:4, 1:8, 0, respectively. Three replicated wells. Twenty-four hours later, cells were fixed and observed as above.

Cell migration assay

The 'wound assay' of Birch was used to determine the migratory capacity. 5×10^4 SMMC7721 or SF7721 cells were planted into the wells of 24-well plates and cultured 24-48 h until the cultures were subconfluent. A wound track was scored in each well. In paracrine system detection, 5×10^4 SMMC7721 were planted into the wells of 24-well plates and cultured 24-48 h until the cultures were subconfluent. A wound track was scored in each well. Conditioned medium was replaced in the experimental group with 1 mL MHCC-1 - CM and 1 mL control medium, control group with 1 mL surum free DMEM and 1 mL control medium. Replicated wells were terminated at 16, 24 and 48 h after wounding and examined under inverted microscope.

Cell growth curve

5×10^4 SMMC7721 or SF7721 were planted into 24-well plates, respectively. From the next day, cells of triplicate wells were digested by typsin and counted under microscope for 8 d. The average was recorded as cell number of the day. In addition, 2×10^4 SMMC 7721 cells were planted into the wells of 96-wells plates. Twenty-four hlater, 200 μL MH CC-1-CM was added into each well within diluted range 1:2, 1:4, 1:8, 0, respectively. Three replicated wells. The effects of MHCC-1-CM on SMMC7721 were also contrasted using SMMC7721-CM. After 72 h incubation, 20 μL MTT (Methabenzthiazuron, Serva Co, USA) was added into each well to a final concentration at 50 g·L-1. Four hours later, the medium with 100 μL DMSO was replaced, and asorbance (OD) was read under 540 nm (Bio-rad).

Inhibition assay by c-met antibody

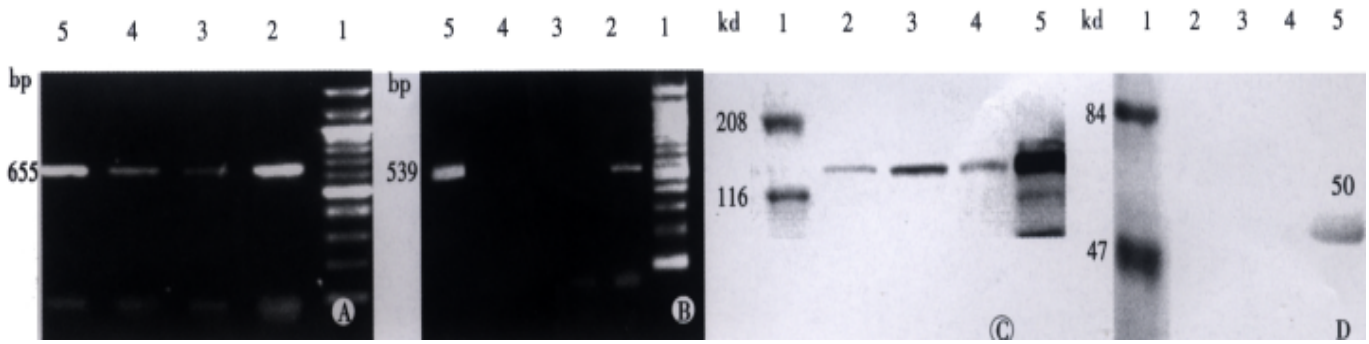
2×10^4 MHCC-1 or S F7721 were planted in 96-well plates, 3 replicated wells. After 24 h incubation, cells were washed by free DMEM, and incubated in 50 μL c-met polyantibody within a concentration range of 20, 10, 5, 2.5, 0 mg·L^{-1}. Rabbit IgG was used as control. Two hours later, 150 ìL control medium was added into each well and 72 h later, cells were examined by MTT. As to SMMC7721, 10^4 cells incubated with 2 μg c-met antibody for 2 h. Afterward, cells were washed by D-Hanks and added into wells of 96-well plates within 100 μL conditioned medium and 100 μL MHCC-1-CM or SM MC7721-CM, respectively. Twenty-four h later, cells were observed under micros cope. Rabbit IgG was used as negative control.

Statistical analysis

In SMMC7721 cell proliferation experiments, Student's *t* test was used to compare the difference of each corresponding dosage groups. Values were expressed as means ± standard. The cell growth curve and c-met blocking assay were tested by two-way anova.

RESULTS

SF and c-met expression in HCC cell lines (Figure 1)



A:c-met transcription B: SF/HGF transcription
1: Mark; 2: MHCC-1; 3: HepG2; 4: Hep3B; 5: SMMC7721; 6: pBS7.3

C: c-met protein expression D: SF/HGF protein expression
1: Mark; 2: SMMC7721; 3: Hep3B; 4: HepG2; 5: MHCC-1

Figure 1 SF/HGF and c-met expression in HCC cell lines.

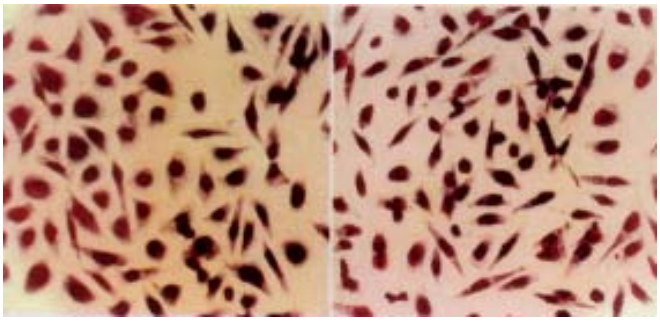
RT-PCR showed that all the cell lines had the transcription and protein expression of c-met, in which MHCC-1 most actively expressed. Only MHCC-1 had the transcription and protein expression of SF/HGF to the medium.

SF/HGF expression in SF7721

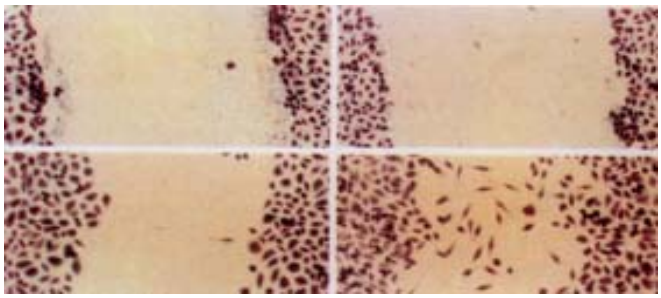
After sf/hgf transfection, in SF7721 cell extracts, the highest expression of SF/HGF reached 692 $\mu g \cdot L^{-1}$, compared with 0.026 $\mu g \cdot L^{-1}$ in control cells. However, no SF/HGF was detected in conditioned medium.

SF/HGF-c-met autocrine stimulate HCC malignancy

After gene transfection, SF7721 cells displayed scattered distribution and elongated morphology (Figure 2A) together with increased ability of proliferation. The cell growth curve showed that after 8 days, cell number of SF7721 reached almost double of that of SMMC7721 (Figure 2C, $P<0.05$). Also, the transfected SF7721 acquired stronger mobility. In "wound healing assay", SF7721 moved faster than SMMC7721 cells into cell free area (Figure 2B). However, the c-met expression in SF7721 and SMMC7721 did not show great difference.



Left:SMMC7721; Right:SF7721
Figure 2A Cell morphology comparison. ×400



Up: Control at "0" time Left down:SMMC7721 at 16 h after wound healing Right down:SF7721 at 16 h after wound helaing
Figure 2 B Cell morphology comparison ×400

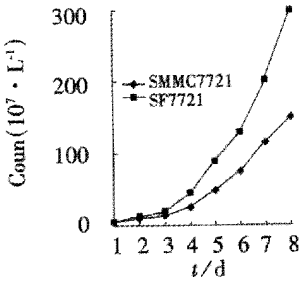


Figure 2 C Cell growth curve before and after gene transfection

Role of SF/HGF-c-met paracrine

The paracrine system of SF/HGF-c-met was examined by stimulating SMMC7721 with MHCC-1-CM. Results were consistent with that in autocrine. The proliferation of SMMC7721 was stimulated by MHCC-1-CM at 1:2 dilution (Table 1, $P<0.01$). In addition, MHCC-1- CM increased the mobility of SMMC7721 cells in wound healing assay and induced the elongated morphology displayed in SF7721 cells. (Data not shown).

Table 1 MHCC-1-CM stimulated proliferation of SMMC7721 (contrasted by SMMC7721-CM, $\bar{x}\pm S_{\bar{x}}$)

Group	Conditioned medium/culture medium (volume ratio) 0	1:16	1:8	1:4	1:2[b]
SMMC7721-CM	0.596±0.090	0.535±0.315	0.568±0.099	0.524±0.053	0.541±0.377
MHCC-1-CM	0.513±0.043	0.581±0.080	0.570±0.061	0.620±0.033	0.853±0.031

[b]$P<0.01$, 0.541±0.377 *vs* 0.853±0.031.

Assay of C-met antibody blocking

Both cell proliferation and motility could be blocked by c-met antibody. After 3-day's incubation with c-met antibody, either MHCC- 1 or SF7721 showed inhibition of growth, and was correlative with antibody concentration (Figure 3, $P<0.05$). As to SMMC7721, the effect of inhibition could be seen under microscope, their shape turned round, and became shrank.

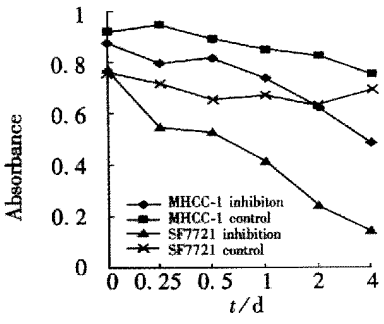


Figure 3 C-met inhibition assay.

DISCUSSION

Metastatic dissemination of solid tumors is a complex patho-physiological process including various factors. When we started to research on a HCC cell line (M HCC-1) with high potential of metastasis, we found that it had a high expression of c-met and with SF/HGF autocrine, which did not exist in other cell lines without or with low potential of metastasis. Thus, it became a promising approach to discuss c-met-HGF/SF signal transduction and tumor metastasis. Our result showed that cell malignancy of HCC is relative to its SF/HGF-c-met expression. The c-met expression of cell line with a higher potential of metastasis is much stronger than those with lower potential of metastasis and appeared with SF/HGF autocrine. Previous studies reported that many cancer cells expressed HGF/SF and c-met *in vivo*, but few of carcinoma cell lines produced HGF/SF *in vitro*, indicating that SF/HGF is a negative regulator in tumor progression. To further eluciate the phenomena, we transfected SF/HGF cDNA into S MMC7 721 cell line, trying to demonstrate that acquired SF/HGF autocrine may increase the malignancy and improve the metastatic potential in less metastatic cells. Our results showed that the proliferation, mobility and cell morphology had greatly changed in

SF7721 cells. Although the c-met expression in SF7721 cell did not increase significantly, it did improved greatly in SF7721 tumors in nude mice assay, later in the *in vivo* research. (Data not shown) When SF/HGF-c-met system was blocked by c-met antibldy, both MHCC-1 and SF7721 were blocked, demonstrating that SF/HGF-c-met was a positive regulator in HCC progression. Thus, we postulated that carcinoma cells may lose the ability to produce HGF/SF during *in vitro* passage, or the expression of HGF/SF need an activation from matrix. The high potential of metastasis of MHCC-1 may, to a great extent, contribute to its preservation of HGF/SF expression and keep the c-met activated all the way. *In vivo*, fibroblasts can produce HGF/SF, which may induce the expression of HGF/SF and c- met in cancer cells, thus establishing an autocrine and paracrine system and promoting cell scatter, proliferation and invasion[37-39]. We also studied the paracrine role of HGF/SF by stimulating SMMC7721 with MHCC-1-CM. Results were cons is tent with that from experiment of autocrine. Cell scatter, proliferation and mobility in SMMC7721 increased after they were stimulated by conditioned medium of M HCC-1. Such biological activities can be blocked by anti c-met polyclonal anti body. However, the influence of SMMC-7721 proliferation by the conditioned medium of MHCC-1 happened only when the medium was in 1:2 dilution, suggesting that the c-met receptor needs a certain amount of SF/HGF to activate. Once activated, the biological activity may depend on the quantity of c-met expression and the ex tent of receptor phosphorylation, thereby initiating downstream regulations. There were two reasons why we choose c-met rather than SF/HGF to be blocked. One was the conflicting reports of SF/HGF. Up to now, various of SF/HGF variants have been found, each having different structure and bioactivity *in vitro* and/or *in vivo*. This could be another reason why different results of SF/HGF are reported[40-47]. Compared with HGF/SF, c-met introduces many biological functions, but all the signal transductions start from a same 'multifunctional docking site'[4,5,48]. The different biological functions come from different signal messages, thus making it a better choice compared with HGF/SF and other members of tyrosine kinase family. Recently, c-met inhibition has become a hot spot in anticancer research[1,2,49-51]. In our research, the tumor cells blocked by c-met antibody shrank in morphology and decreased in cell proliferation. Results suggested that the inhibition of met-SF/HGF could become one of the potential approaches to reduce tumor growth and metastasis. In conclusion, our experiment showed that the system of SF/HGF-c-met autocrine and paracrine play an important role in invasion and metastasis of hepatocellular carcinoma. Inhibition of c-met-H GF/SF system may reduce the proliferation and metastasis of hepatocellular carcino ma by lowering the expression of c-met or its downst ream signal transduction.

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