Involvement of c-Met/Hepatocyte Growth Factor Pathway in Cholangiocarcinoma Cell Invasion and Its Therapeutic Inhibition With Small Interfering RNA Specific for c-Met

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Background. Hepatocyte growth factor receptor (c-Met) plays an important role in many functions of cancer cells. We examined the roles of c-Met and its downstream signaling molecules in cholangiocarcinoma cell lines RMCCA1 and HuCCA1.

Materials and methods. The expression of c-Met and their signaling cascades were determined in RMCCA1 and HuCCA1 cholangiocarcinoma cell lines by Western blotting. Small interfering RNA (siRNA) specific for c-Met was used to suppress the expression of c-Met. The proliferation, migration and invasion assay were tested in these cholangiocarcinoma cells treated with hepatocyte growth factor (HGF).

Results. Activation of c-Met with HGF triggered the signaling via the ERK cascade mediated by sequential phosphorylation of MEK1/2 and MAPK and induction of cholangiocarcinoma cell invasion. The expression of c-Met in cholangiocarcinoma cells was suppressed by treatment with small interfering RNA (siRNA) specific for c-Met, and resulted in decrease in phosphorylation of MEK1/2. Furthermore, treatment with siRNA specific for c-Met or MEK inhibitor U0126 inhibited cholangiocarcinoma cell invasion induced by HGF.

Conclusions. These results indicated that HGF and c-Met involved in the mechanism of cholangiocarcinoma cell invasion. It implies a potential role for the inhibition of c-Met in the treatment of cholangiocarcinoma. © 2006 Elsevier Inc. All rights reserved.

Key Words: cholangiocarcinoma; c-Met; migration; invasion; MEK1/2.

INTRODUCTION

Cholangiocarcinoma is a cancer arising from bile duct epithelium. It is one of the most difficult diseases to treat. Three-year survival rates of 35 to 50% can be achieved in only a few numbers of patients when negative histological margins are attained at the time of surgery [1]. The reason for this poor prognosis is that cholangiocarcinoma exhibits extensive local invasion and frequent regional lymph node metastasis [2]. In addition, there is no effective chemotherapeutic drug to kill cholangiocarcinoma cells.

c-Met (hepatocyte growth factor receptor) is a tyrosine kinase receptor for hepatocyte growth factor (HGF). The previous study demonstrated that the over expression of c-Met has been found in many kinds of cancers [3–6] including cholangiocarcinoma [7]. Stimulation of c-Met activates multiple signal intermediates such as PI3k and MEK1/2 that has been recognized as the key factors influencing the events of tumor invasion and metastasis [8–10]. Therefore, the pharmacologic intervention that effects c-Met expression and its signal transduction may influence the invasiveness of cholangiocarcinoma cell.

In this study, we have demonstrated that the stimulation of c-Met with HGF induced cholangiocarcinoma cell invasion. In addition, inhibition of c-Met expression with siRNA or inhibition of its signal transduction intermediate molecule (MEK1/2), suppressed the invasiveness of cholangiocarcinoma cell.

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MATERIALS AND METHODS

Cell Culture and Materials

Hams F12 medium and fetal bovine serum were purchased from Gibco (Gibco, Grand Island, NY). The recombinant human HGF and 24-well Biocoat Matrigel invasion chamber (8 μm) were purchased from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ). Anti-c-Met, anti-MEK, anti-Phospho-MEK1/2 (Ser217/221), anti-MAPK and anti-Phospho-MAPK antibodies were purchased from Cell Signaling (Cell Signaling Technology, Beverly, MA). The human cholangiocarcinoma cell line HuCCA1 (kindly provided by Prof. Sirisinha, Department of Microbiology, Mahidol University) and RMCCA1 (established from Department of Surgery, Rajavithi Hospital) were grown in HAMS F12 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ humidified atmosphere.

Small Interfering RNA (siRNA) for Inhibition of c-Met Expression

Three stealth small interfering RNA (siRNA) duplex oligoribonucleotides specific for c-Met (NCBI Ref Seq NM_000245) were synthesized. The sequences were as follows: 1) MET-HSS106477 sense 5'-UUAAACGCAAACUCGUAGAUAAGC-3', antisense 5'-CCAUUUCAACUGAGUUUGCUGUAA-3'; 2) MET-HSS106478 sense 5'-UGAUUAGGAACGUAGUCCCGGA-3', antisense 5'-UCCAGAAGACGCUUUCCUAUAUC-3'; 3) MET-HSS106479 sense 5'-AACAAUUUCAGUAUGUCUCGGC-3', antisense 5'-CCGAAGGAAUCUAGAAAGAUU-3'. siRNA oligos were transfected into cholangiocarcinoma cells by using BLOCK-it transfection kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The BLOCK-it fluorescent oligo that is not homologous to any known genes was used as transfection efficiency detector and a negative control to ensure against induction of non-specific cellular events caused by introduction of the oligo into cells. Among the three siRNA oligo duplexes specific for c-Met, the one that required the smallest concentration to achieve the desired knockdown effect was selected and used in all experiments. The transfected cells were subjected to proliferation, migration, and invasion assay. The mRNA inhibiting levels were assessed by Real time reverse transcription-polymerase chain reaction (RT-PCR) and the protein inhibiting levels were analyzed with Western blotting after 48 h of treatment.

Quantitative Real-Time RT-PCR and Western Blotting Analyses

Total RNA was isolated from cells by RNeasy kit (Qiagen, Inc., Valencia, CA). Quantitative real-time RT-PCR was performed with Quantitect SYBR Green RT-PCR (Qiagen, Inc.) in a standard PCR reaction mixture. The amplification primers were: 1) c-Met: forward primer 5'-ACAAGTGGCATGTCAACATCGCT-3' and reverse primer 5'-TCCATGACAACTTTGGTATCG-3'; 2) GAPDH: forward primer 5'-TCCATGACAACTTTGGTATCG-3' and reverse primer 5'-GTCGCTGTGTTGAATGCAGAGA-3'. Amplification and detection were performed in a BIO-RAD iCycler iQ system (Bio-Rad, Hercules, CA). The fluorescence threshold value was calculated using the iCycler iQ system software. The conditions were started with; 20 min., 50°C. Then 42 cycling steps for amplification of PCR products were as follow; 15 s, 94°C for denaturation, 30 s, 60°C for annealing and 30 s, 72°C for extension. A single fluorescence measurement was taken at each extension step. The crossing points (Cp), marking the cycle when the fluorescence of a given sample significantly exceeded the baseline signal were recorded and expressed as a function of the cycle number. The melting curve analysis was performed to assess the specificity of the amplified products. The concentration of PCR product was calculated based on established standard curve derived from serial dilutions of the positive controls for GAPDH and c-Met in the HuCCA1 cells. For Western blot analysis, 5 x 10^5 cells were seeded in a six-well culture plate, followed by treating with 40 ng/mL HGF for 15 min. Cells were collected and then Western blot analyses were performed as previously described [11]. Antibodies against c-Met, Phospho-Met, MEK1/2, Phospho-MEK1/2 (Ser217/221), MAPK, Phospho-MAPK, Akt, Phospho-Akt, and actin obtained from Cell Signaling (Cell Signaling Technology, Beverly, MA) were used at a dilution of 1:1000. Chemiluminescence detection of antibody-antigen complexes revealed the target proteins on X-ray film.

Cell Proliferation Assay

Cells were seeded in 96-well culture plates at a density of 1 x 10^4 cells per well followed by the addition of HGF in various concentrations. For MEK inhibition study, cells were treated with U0126 (Cell Signaling Technology) [12] for 1 h before addition of HGF. Then cells were incubated for 1 to 3 days before applying the WST-1 cell proliferation assay reagent (Roche Diagnostics, Laval, Quebec, Canada) according to the recommendation of the manufacturer. Percentage of proliferation was calculated based on untreated cells.

Cell Migration Assay

Migration of cholangiocarcinoma cells was assayed using chamber with 8-μm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). There were 5 x 10^4 cholangiocarcinoma cells added to the upper chamber. Then 0.5 mL serum-free media with 40 ng/mL of HGF was added to the lower chamber. The chambers were incubated for 12 h at 37°C. After incubation, the filters were fixed and stained with hematoxylin and counted in five random high-power fields under a light microscope.

Cell Invasion Assay

The invasion of cholangiocarcinoma cells was assayed in 24-well Biocoat Matrigel invasion chamber (8 μm; Becton Dickinson). There were 5 x 10^4 cells seeded in the upper chamber. The bottom chamber contained 40 ng/mL of HGF. After 24 h of incubation, the invading cells at the lower surface of Matrigel-coated membrane were fixed with 70% ethanol, stained with hematoxylin and counted in five random 100 X power fields under a light microscope.

Detection of Actin Cytoskeleton

Cholangiocarcinoma cells were treated with siRNA specific to c-Met or control, seeded on coverslips and incubated for 24 h. Then the cells were incubated in serum-free medium containing with 40 ng/mL of HGF for 4 h. The cells were fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100 for 15 min and blocked with 1% BSA. The cells were exposed to Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR) for 30 min and washed with TTBS. Then coverslips were mounted on the slide-glass using 50% glycerol in PBS. The cells were examined under a fluorescent microscope (Olympus).

Statistical Analysis

The experiments were all performed in triplicate and identical results were obtained. Values were expressed as the mean and SD. The student’s t-test was used for analysis of the cell proliferation and invasion assay. The P value of less than 0.05 was considered significant.

RESULTS

Expression of c-Met in Cholangiocarcinoma Cells

The expression of c-Met in two cholangiocarcinoma cell lines (RMCCA1 and HuCCA1) was investigated. Western
blot analysis demonstrated definite expression of c-Met in both cholangiocarcinoma cell lines (Fig. 1).

**The Effect of HGF on Cholangiocarcinoma Cell Proliferation**

Because the activation of c-Met with HGF was known to play an important role in cell proliferation in many kinds of cancer cells, we investigated the role of HGF in cholangiocarcinoma cell proliferation. Cell proliferation assay was performed in HuCCA1 and RMCCA1 cells treated with HGF at concentrations of 0, 20, 40, and 100 ng/mL. After 3 days, the results showed that HGF had no effect on cholangiocarcinoma cell proliferation (Fig. 2).

**The Effect of HGF on Cholangiocarcinoma Cell Migration and Invasion**

To study the mechanism by which HGF induced the migration of cholangiocarcinoma cells, cell migration assay was performed. We found that HGF induced the migration of RMCCA1 and HuCCA1. Their maximum effect was identified at 40 ng/mL of HGF. The maximal migration indices were 250 ± 20%. To test whether HGF induced cholangiocarcinoma cell invasion, standard invasion assay was performed with RMCCA1 and HuCCA1 cells treated with HGF at the concentration of 40 ng/mL. HGF enhanced cholangiocarcinoma cell invasion more than two-fold compared with untreated cells (Fig. 3).

**The Effect of HGF on the Phosphorylation of MEK1/2 in Cholangiocarcinoma Cells**

We attempted to evaluate the signaling pathways relevant to HGF-induced invasion of cholangiocarcinoma cell. The phosphorylation of signal molecules, which was previously demonstrated as c-Met, mediated signaling molecules, was assayed by Western blot analysis. RMCCA1 and HuCCA1 cells were treated with HGF and then the cell lysate was used for detection of the phosphorylation of MEK1/2, and MAPK. HGF-treated cells demonstrated a higher extent of the phosphorylated MEK1/2 and MAPK than untreated cells (Fig. 4). However, the phosphorylated Akt was detected only in HGF-treated RMCCA1 cells.
The Effect of c-Met siRNA on the Expression of c-Met and the Effect of c-Met siRNA on HGF-Induced Phosphorylation of MEK1/2 in Cholangiocarcinoma Cells

To determine whether the activation of c-Met and its signal transduction MEK1/2 are necessary for cholangiocarcinoma cell invasion, cells were transfected with siRNA targeted to c-Met. The expression of c-Met mRNA was detected by real time RT-PCR and Western blot analysis. The significant suppression of c-Met was identified in HuCCA1 and RMCCA1 cells transfected with c-Met siRNA at 48 h comparing with control siRNA (Fig. 5). The HGF-induced phosphorylation of MEK1/2 was also investigated after treated the cells with c-Met siRNA or control siRNA. The phosphorylation of MEK1/2 in c-Met siRNA treated cells was extensively lower than in control siRNA treated cells (Fig. 6).

The Effect of c-Met siRNA or MEK1/2 Inhibitor U0126 on HGF-Induced Cholangiocarcinoma Cell Proliferation and Cell Invasion

Activation of c-Met with HGF had no effect on HuCCA-1 and RMCCA1 cell proliferation. Furthermore, the treatment of U0126 or c-Met siRNA to cholangiocarcinoma cells did not show inhibitory effect on cell proliferation (Fig. 7).

To study the mechanism by which c-Met siRNA or U0126 inhibited the HGF-induced invasion of cholangiocarcinoma cells. HuCCA1 and RMCCA1 cells were transfected with c-Met siRNA or U0126 and then treated with HGF for evaluation of cell invasion activity. Cells transfected with control siRNA were used as a control. The invasion induced by HGF was inhibited by c-Met siRNA or U0126 in both cholangiocarcinoma cell lines (Fig. 8).

The Effect of HGF on Actin Cytoskeleton of Cholangiocarcinoma Cells

Serum-starved cells showed low levels of actin polymerization. After the treatment with 40 ng/mL of HGF, RMCCA1 cells displayed high levels of actin polymerization in the peripheral of the cells and a distinct pseudopodia formation. Treatment of cholangiocarcinoma cells with c-Met siRNA before addition of HGF caused eradication of actin polymerization (Fig. 9).

DISCUSSION

Cholangiocarcinoma is one of the dismal diseases characterized by early vascular invasion and distant metastasis. Although the molecular biology of chol-
angiocarcinoma has been studied, the molecular mechanism for cholangiocarcinoma cell invasion and metastasis has to be defined further. c-Met is a tyrosine kinase receptor that is expressed in many kinds of cancers and activated by its ligand HGF expressed on mesenchymal cells [9]. The previous studies suggested that activation of c-Met resulted in many physiological activities of cells including induction of angiogenesis, cell proliferation and cell invasion [3, 4, 6]. In addition, up-regulation of c-Met by retinoic acid can induce cancer cell invasion [11].

In this study, the effect of HGF on c-Met in RMCCA1 and HuCCA1 cholangiocarcinoma cells was tested by using cell proliferation assay, cell invasion assay and siRNA suppression of c-Met expression. The findings provided several data about the significant molecules that promote cholangiocarcinoma cell invasion. We identified that cholangiocarcinoma cells express c-Met both RNA level and protein level and stimulation of c-Met with HGF promotes cancer cell migration and invasion. Our

**FIG. 6.** The effect of HGF and c-Met siRNA on the phosphorylation of MEK1/2. (A) RMCCA1 and HuCCA1 were transfected with the c-Met siRNA and control siRNA for 48 h and then treated with 40 ng/mL of HGF for 15 min. MEK1/2 phosphorylation (P-MEK) was determined by Western blot. c-Met siRNA transfection significantly inhibited the phosphorylation of MEK1/2 induced by HGF. (B) Results from three biologically separate experiments, showing mean levels of P-MEK expression ± SD resulting from c-Met siRNA transfection relative to levels from cells transfected with control siRNA.

**FIG. 7.** The effect of HGF and MEK1/2 inhibitor (U0126) on cell proliferation in HuCCA1 cells transfected with c-Met siRNA and control siRNA. RMCCA1 and HuCCA1 transfected with the c-Met siRNA and control siRNA for 48 h were treated with and without U0126 and then stimulated with 40 ng/mL of HGF for 24 h. Cell proliferation assays were determined by WST-1. The data represent the average results from three individual experiments.
studies suggested that these events involved the activation of the extracellular signal-regulated kinase (ERK) cascade, a central pathway that transmits signals from many extracellular agents to regulate cellular processes. The latter assertion is based on the finding that inhibition of c-Met expression by siRNA or inhibition of MEK1/2 by its specific inhibitor U0126 suppressed the phosphorylation of MEK1/2 and also inhibited the invasiveness property of cholangiocarcinoma cell. MEK1/2 is dual specificity protein kinases that function in a mitogen activated protein kinase cascade controlling cell growth and differentiation [13]. Activation of MEK1/2 occurs to phosphorylation of two serine residues at position 217 and 221 (in the activation loop of subdomain VIII) by Raf-like molecules. MEK1/2 is activated by a wide variety of growth factors and cytokines, and also by membrane depolarization and calcium influx [14]. Although, some reports suggested that HGF was a potent stimulator for Akt phosphorylation and cancer cell proliferation [15], other reports including this study made exactly opposite conclusions [4]. Stimulation of c-Met with HGF has neither effect on Akt phosphorylation nor cell proliferation in HuCCA1 cells. Evidence existed that the effect of c-Met to enhance cancer cell proliferation may need the activation of PI3-kinase pathway which was not defined in HuCCA1 cells. The different biological functions may come from different activations of the multifunctional docking site of c-Met.

In cancer cells, high levels of actin polymerization are important for the formation of pseudopodia, which in turn are implicated in the enhancement of cancer cell migration and invasion [16]. This study showed that the treatment of cholangiocarcinoma cells with HGF resulted in the increase in actin polymerization. In addition, inhibition of c-Met expression with c-Met siRNA resulted in a dramatic decrease in action polymerization. These findings suggested that HGF and c-Met plays an important role in the invasion as well as the metastasis in cholangiocarcinoma.

In conclusion, this experiment showed that the stimulation of c-Met plays an important role in cholangiocarcinoma cell invasion. Inhibition of c-Met and its pathway could become one of the potential approaches for cholangiocarcinoma therapy.

**FIG. 8.** The suppression of migration and invasion activity of cholangiocarcinoma cells after treatment with c-Met siRNA, control siRNA, and MEK inhibitor (U0126). (A) RMCCA1 and HuCCA1 transfected with the c-Met siRNA and control siRNA for 48 h then were treated with and without U0126. There were $5 \times 10^3$ cells seeded in the 8-µm pore filters (Transwell, 24-well cell culture, Coster, Boston, MA). The bottom chamber contained 40 ng/mL of HGF. After 24 h, the cells on the lower surface were counted under a microscope at five random 100× power fields. The experiment was repeated for three times and the data represent the average results from three individual experiments. (B) RMCCA1 and HuCCA1 transfected with the c-Met siRNA and control siRNA for 48 h then were treated with and without U0126. There were $5 \times 10^3$ cells seeded in the 24-well Biocoat Matrigel invasion chamber. The bottom chamber contained 40 ng/mL of HGF. After 24 h, the cells on the lower surface were assayed as previously described.

**FIG. 9.** Effect of HGF and c-Met on actin cytoskeleton in RMCCA1 cells. RMCCA1 cells were transfected with control siRNA or c-Met siRNA and then treated with 40 ng/mL HGF. The cells were stained with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR) to visualize actin cytoskeleton. (Color version of figure is available online.)
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