Hepatocyte Growth Factor Promotes Cancer Cell Migration and Angiogenic Factors Expression: A Prognostic Marker of Human Esophageal Squamous Cell Carcinomas

Yi Ren,1 Brian Cao,2 Simon Law,1 Yi Xie,1 Ping Yin Lee,1 Leo Cheung,1 Yongxong Chen,1 Xin Huang,1 Hiu Man Chan,1 Ping Zhao,2 John Luk,1 George Vande Woude,2 and John Wong1

Abstract

Purpose: Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, c-Met, play important roles in tumor development and progression. In this study, we measured the serum HGF levels in patients with esophageal squamous cell carcinoma (ESCC) to evaluate its relationships with clinicopathologic features and the role of HGF in ESCC.

Experimental Design: One hundred and forty-nine patients with ESCC were studied. Pretherapy serum was collected and ELISA was used to detect the concentrations of HGF, vascular endothelial growth factor (VEGF), and interleukin 8 (IL-8). The function of HGF was shown by invasion chamber assay.

Results: Pretherapy serum HGF was found to be significantly higher in patients with ESCC than in control subjects. The levels of HGF correlated significantly with advanced tumor metastasis stage and survival. Multivariate analyses showed that serum HGF level in cell migration was an independent prognostic factor. Increased HGF serum levels correlated positively with serum levels of VEGF and IL-8. Our results also showed that HGF was overexpressed in ESCC tissues and cell lines. In vitro study showed that HGF could stimulate ESCC cell to express VEGF and IL-8 and markedly enhance invasion and migration of ESCC cells. Furthermore, HGF-induced IL-8 and VEGF expression was dependent on extracellular signal-regulated kinase signaling pathways. The inhibition of extracellular signal-regulated kinase activation reduced HGF-mediated IL-8 and VEGF expression.

Conclusions: Our results suggest that serum HGF may be a useful biomarker of tumor progression and a valuable independent prognostic factor in patients with ESCC. HGF may be involved in the progression of ESCC as an autocrine/paracrine factor via enhancing angiogenesis and tumor cell invasion and migration.

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive and lethal malignancies (1). Overall, the 5-year survival rate of patients with esophageal cancer is around 10% to 20%. Even with apparently curative surgical resection, this figure approximates only 40%. Several recent studies have shown that tumor-node-metastasis stage, completeness of surgical resection (in those with resectable tumors), and the number of diseased lymph nodes are the most important prognostic factors (2). Although various molecular biological factors, such as vascular endothelial growth factor (VEGF; ref. 3), p53 (4), cyclin D1 (5), Bcl-2 (6), and E-cadherin (7), have been proposed as prognostic indicators for ESCC, more refined and accurate variables that predict survival are lacking. New indicators of the malignant potential of ESCC are particularly desired. Understanding the molecular mechanisms of the complex multistep process of esophageal tumorigenesis could facilitate the development of preventive measures, early diagnostic methods, and better treatments. Despite intensive investigations, the fundamental role of tumor initiation and progression in ESCC remains elusive.

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchymal cytokine with a number of biological activities, including mitogenic, motogenic, and/or morphogenic properties, in a variety of epithelial tissues. HGF is also a known angiogenesis factor by its ability to promote endothelial cell growth, survival, and migration both in vitro and in vivo (8, 9). HGF was originally detected in the blood of partially hepatectomized rats and rat platelets (10, 11). It achieves its effect primarily in a paracrine fashion. c-Met, a proto-oncogene,
Significance of Serum HGF in Esophageal Cancer

Materials and Methods

Reagents. All reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated. All antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Patients and serum samples. One hundred and forty-nine patients with ESCC treated at the Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital between January 1997 and August 2003 were recruited in this study. All patients had squamous cell carcinomas of the thoracic esophagus but no history of any other cancers. There were 124 men and 25 women, with a median age of 66 years. Sixty-five (43.6%) patient tumors were treated with surgical resection upfront, 55 (36.9%) patients underwent neoadjuvant chemoradiation therapy before surgery, and 29 (19.5%) had chemoradiation therapy only. Blood samples were collected from all patients before treatment and from 35 control subjects. These were either healthy volunteers or patients with unrelated problems with no history of malignancy. Sera were collected by centrifuging the whole blood at 500 × g for 15 minutes, and the sera aliquots were stored at −80°C until used in ELISA assay. This study was approved by the Human Research Ethics Committee, Queen Mary Hospital, University of Hong Kong. Informed consent was obtained according to the regulations of the committee.

Measurement of the concentrations of hepatocyte growth factor in the sera of patients with esophageal squamous cell carcinoma and in the supernatants of tumor cell culture. HGF in the sera of patients with ESCC and in the supernatants of tumor cell culture was measured using ELISA. After washing with wash buffer [PBS with 0.05% Tween (pH 7.4)] thrice, antihuman HGF monoclonal antibodies coated plate was blocked with blocking buffer [PBS with 1% bovine serum albumin (pH 7.4)] at 4°C overnight. Serum samples and HGF standard were added into each well of plate and incubated at room temperature for 2 hours. After being washed, the plate was incubated with conjugated second antibody for another 2 hours, and thereafter was washed again and substrate solution added. The color reaction was measured by absorbance at 405 nm using a photospectrometer. All samples and standards were assayed in duplicate.

Measurement of the concentrations of vascular endothelial growth factor and interleukin-8 in sera of patients with esophageal squamous cell carcinoma and in the supernatants of tumor cell culture. Levels of VEGF and IL-8 in sera of patients and healthy subjects and in the

Table 1. Relationship between serum HGF levels and clinicopathologic features

<table>
<thead>
<tr>
<th>Variables</th>
<th>Serum HGF (pg/mL)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 124)</td>
<td>605.6 (37-4,614)</td>
<td>0.54</td>
</tr>
<tr>
<td>Female (n = 25)</td>
<td>588.0 (27-1,163)</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
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<td></td>
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<tr>
<td>&lt;65 (n = 70)</td>
<td>637.5 (37-4,614)</td>
<td>0.22</td>
</tr>
<tr>
<td>≥66 (n = 79)</td>
<td>569 (27-2,513)</td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (n = 5)</td>
<td>517 (262-717)</td>
<td>0.47</td>
</tr>
<tr>
<td>T2 (n = 16)</td>
<td>579.5 (69-1,060)</td>
<td></td>
</tr>
<tr>
<td>T3 (n = 115)</td>
<td>618.3 (27-6,461)</td>
<td></td>
</tr>
<tr>
<td>T4 (n = 13)</td>
<td>643.0 (287-1,418)</td>
<td></td>
</tr>
<tr>
<td>Node stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0 (n = 41)</td>
<td>569 (27-1,163)</td>
<td>0.10</td>
</tr>
<tr>
<td>N1 (n = 108)</td>
<td>626.5 (37-4,641)</td>
<td></td>
</tr>
<tr>
<td>Metastasis stage</td>
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<tr>
<td>M0 (n = 111)</td>
<td>558 (27-2,513)</td>
<td>0.02*</td>
</tr>
<tr>
<td>M1a/1b (n = 38)</td>
<td>764.1 (99.3-4,641)</td>
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</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I/II (n = 40)</td>
<td>496.5 (27-1,163)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Stage III/IV (n = 109)</td>
<td>632.0 (37-4,641)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Serum HGF levels are expressed as median (range).
*P < 0.05 by Mann-Whitney U test.
supernatants of tumor cell culture were measured using ELISA kits (R&D Systems, Minneapolis, MN). ELISA was done according to the instructions of the manufacturer.

**Immunohistochemical staining.** One-color immunohistochemical staining using monoclonal antibodies to HGF and c-Met was done as described previously (31). Paraffin sections were incubated with monoclonal antibodies overnight at 4°C. Sections were then washed with PBS (pH 7.4). Endogenous peroxidase was inactivated in 3% H2O2/C2H4tetrazolium/X-phosphate. Antidigoxigenin F(ab) fragments and color development with nitroblue tetrazolium/X-phosphate. Purified rabbit nonspecific IgG was used as a negative control.

**In situ hybridization.** In situ hybridization was done on paraffin sections (4 μm) of formalin-fixed tissue. Sections were treated by heating in a microwave oven, incubated with 0.2 mol/L HCl for 15 minutes, followed by 1% Triton X-100 for 5 minutes, and finally digested with 10 μg/ml protease K (Boehringer Mannheim GmbH, Mannheim, Germany) at 37°C for 20 minutes. After being washed in 2× SSC, the sections were prehybridized and then hybridized with digoxigenin-labeled sense or antisense HGF cRNA probe (2.3 kb) overnight at 42°C in a hybridization buffer containing 50% deionized formamide, 4× SSC, salmon spermin DNA (1 mg/ml), and yeast tRNA (1 mg/ml). Sections were then washed in 0.1× SSC at 42°C and the hybridized probe was detected using alkaline phosphatase- conjugated sheep antidigoxigenin F(ab) fragments and color development with nitroblue tetrazolium/X-phosphate.

**Cell lines.** Three previously reported human ESCC cell lines, HKESC-1, HKESC-2, and SLMT, were established from primary ESCC from Hong Kong Chinese patients (32–34). Normal epithelial cell line (NE1) was established from normal esophageal tissue immortalized by virus. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) and antibiotic agents (100 units/ml penicillin G and 80 μg/ml streptomycin, Life Technologies).

**Cell stimulation assay.** For stimulation experiments, cells were seeded at 5×10⁴/ml in 24-well plates and then cultured in DMEM with 10% fetal bovine serum for 24 hours. Cells were washed with PBS and treated with DMEM supplemented with 1% bovine serum albumin and different concentrations of recombinant human HGF (ranging from 1 to 50 ng/ml) for 24 hours. The culture supernatants of cells were collected after each experiment for ELISA.

**Migration assay.** Tumor cell invasion and migration were assayed in an invasion chamber (Becton Dickinson Labware, Becton Dickinson, Bedford, MA) with 12 μm porosity polycarbonate filter membrane (31). The insert coated with Matrigel matrix was placed in each well of a 24-well plate filled with 500 μl of medium (DMEM with 1% fetal bovine serum) in the presence of various amounts of HGF. The upper well contained suspension of HKESC cells or control cell line NE1 (200 μl, 1×10⁴ cells/ml/medium). The cells in chambers were incubated for 4 hours at 37°C, and migration to the underside of the precoated filter was measured. After incubation at 37°C for 24 hours, the cells on the upper surface of the filters were removed by swabbing with a cotton swab and the cells that had migrated to the lower surface were counted under a microscope. For each chamber, the number of migrated cells in five randomly chosen high-power fields was counted. All assays were done in triplicate and at least three independent experiments were done.

**Western blot.** Cells were washed with PBS and directly lysed in RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, and 1% NP40 and PBS) with PMSF and protease inhibitors. The samples were adjusted to equal protein concentrations and subjected to SDS-PAGE. Proteins on the gel were transferred onto nitrocellulose membranes that were blocked with 5% bovine serum albumin in TBS containing 0.1% Tween 20 for 1 hour at room temperature. Afterwards, the membranes were incubated with the indicated primary antibodies overnight at 4°C. After being washed with TBS containing 0.1% Tween 20, the membranes were incubated with the appropriate secondary antibody. The immunoreactive bands were visualized with chemiluminescence (ECL, Amersham, Boston, MA).

**Statistical analyses.** Continuous variables were expressed as median and range. Differences in medians were compared by Mann-Whitney U test. Kendall’s τ-b test was used as measurement of correlation between variables. The contribution/impact of serum HGF level and clinicopathologic parameters on survival was studied by Cox regression model. Statistical significance was taken as P < 0.05. All statistical analyses were done using the SPSS software for windows 8.0 (SPSS, Inc., Chicago, IL).

**Table 2. Multivariate analysis with Cox proportional hazards model**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Hazard ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum HGF level</td>
<td>1.0009 (1.0005–1.0014)</td>
</tr>
<tr>
<td>Preoperative staging</td>
<td>1.5899 (1.1064–2.2846)</td>
</tr>
<tr>
<td>Resection</td>
<td>0.2240 (0.1133–0.4429)</td>
</tr>
<tr>
<td>Chemoradiation</td>
<td>0.3887 (0.2181–0.6929)</td>
</tr>
</tbody>
</table>

**Table 3. Serum levels of cytokines in patients with ESCC and in control subjects**

| Cytokine | Patients (pg/mL) | Healthy controls (pg/mL) | P
|----------|------------------|--------------------------|---
| HGF      | 600 (27-4,614)   | 214 (43.6-248.0)         | <0.001
| IL-8     | 20.0 (10.3-41.1)| 1.0 (0.2-3.7)            | <0.001
| VEGF     | 2,380.0 (1,120.0-3,390.0)| 140.0 (41.0-230.0) | <0.001

Note: HGF was tested in 35 control subjects and IL-8 and VEGF in 15, all assayed for each cytokine by ELISA. P < 0.05 by Mann-Whitney U test.
Results

Serum hepatocyte growth factor levels and clinicopathologic features. The median pretherapy serum HGF level of the 149 patients was 600 pg/mL (27.4-614) compared with that of 214 pg/mL (43.6-248) in control subjects (P < 0.001; Fig. 1). The relationships between serum level of HGF and clinicopathologic features are shown in Table 1. There was no significant correlation between serum HGF concentration and patients’ gender, age, and tumor and node stage. However, serum HGF levels were significantly higher in patients with M1a/b disease, and patients whose tumor was at stage III or IV also had higher HGF level compared with those who had stage I or II disease.

Serum hepatocyte growth factor levels and survival. Taking the median serum HGF level (600 pg/mL) in the whole group as the cutoff value, patients were divided into high (>600 pg/mL) and low serum HGF (≥600 pg/mL) groups. The median survival of patients with low serum HGF levels (n = 75) was 34.7 months, and was significantly better than that of patients with high HGF levels (n = 74) at 15.8 months (P = 0.014). The respective 2-year survival figures were 63.5% versus 37.0% (Fig. 2).

Prognostic value of serum hepatocyte growth factor. Multivariate analysis using the Cox regression model was used to analyze independent factors of prognostic value. Variables tested included age, gender, pretherapy tumor-node-metastasis stage, overall pretherapy tumor-node-metastasis stage, type of main treatment (resection versus nonoperative treatment), and use of chemoradiation therapy. Factors that were identified as independent prognostic factors are shown in Table 2. Adverse factors included high HGF level and advanced pretherapy stage, whereas surgical resection (versus no resection) and the use of chemoradiation were favorable variables.

Correlations between serum hepatocyte growth factor and other angiogenic factors. Serum levels of VEGF and IL-8 were also evaluated in ESCC patients and control subjects (Table 3). The
levels of HGF, VEGF, and IL-8 in the sera of patients were all significantly higher compared with healthy subjects. Furthermore, the level of serum HGF correlated positively with the serum levels of VEGF ($r = 0.299$, $P < 0.001$) and IL-8 ($r = 0.263$, $P < 0.001$), respectively, suggesting that HGF, VEGF, and IL-8 expression were related.

Expression of hepatocyte growth factor and c-Met in esophageal squamous cell carcinoma specimens. Protein expression of HGF and c-Met in tissue specimens in 15 patients was assayed by immunohistochemical staining. c-Met expression was observed in tumor cells in all specimens from patients with ESCC (Fig. 3A) but it was negative in nontumor tissue (Fig. 3B). There were two patterns of HGF expression. HGF expression was frequently detected in tumor cells in some specimens from patients with ESCC (Fig. 3C), whereas in others only stromal cells such as fibroblasts, endothelial cells, and infiltrated cells (Fig. 3D) expressed HGF. By using in situ hybridization, we found HGF mRNA expression patterns were similar to HGF protein expression patterns (Fig. 3E and F).

Expression of hepatocyte growth factor and c-Met in human esophageal squamous cell carcinoma cell lines. The amount of secreted HGF in the supernatants of cultured ESCC cell lines was measured by ELISA after 24 hours of incubation. ESCC cell lines HKESC2 and SLMT secreted large amount of HGF in the cell culture supernatants, whereas ESCC cell line HKESC1 and normal epithelial cells NE1 expressed very limited HGF (Fig. 4). c-Met expression in all ESCC cell lines and NE-1 was positive (data not shown).

Effect of hepatocyte growth factor on stimulation of vascular endothelial growth factor and interleukin-8 secretion in esophageal squamous cell carcinomas. To detect whether HGF contributes to the production of VEGF and IL-8 by ESCC cells, NE1 and HKESC1 were stimulated with HGF for 24 hours. IL-8 and VEGF in the supernatants of cultured cell lines were measured by ELISA. HGF at concentrations up to 100 ng/mL induced a significant increase of IL-8 and VEGF secretion by HKESC1 in a dose-dependent manner (Fig. 5). However, HGF had no effect on normal epithelial cell line NE1.

Hepatocyte growth factor–induced mitogen-activated protein kinases in esophageal squamous cell carcinomas. As HKESC-1 and NE1 expressed similar levels of c-Met, we measured...
phospho–c-Met in these two cell lines. As shown in Fig. 6A, c-Met phosphorylation in HKESC-1 was stronger compared with NE-1. It may partially explain the differential expression of IL-8 and VEGF in response to HGF treatment between NE-1 and HKESC-1.

Because the mitogen-activated protein kinase pathway cascade is an important signaling pathway that is activated by various growth factors, we evaluated the time dependence of HGF-induced mitogen-activated protein kinase activity in ESCC cells. Western blot analysis showed that HGF at 100 ng/mL rapidly phosphorylated p42/44 extracellular signal-regulated kinase (ERK), reaching a maximum within 30 minutes (Fig. 6B). The total 42/44 ERK protein level remained unchanged. To determine whether mitogen-activated protein kinase activation played a role in HGF-induced IL-8 and VEGF expression, cells were pretreated with ERK inhibitor PD98059 for 60 minutes before HGF stimulation. As shown in Fig. 6C, pretreatment with PD98059 impaired HGF-induced p42/44 ERK phosphorylation. Consequently, HGF-induced IL-8 and VEGF expression was inhibited by PD98059 in a dose-dependent manner (Fig. 7).

Effect of hepatocyte growth factor on hepatocellular carcinoma cell migration. To assess the role of HGF on ESCC cell migration, migration of ESCC cells in response to HGF was examined by invasion chamber method. Various doses of HGF from 5 to 50 ng/mL were added in the lower chambers. Migration of ESCC cells was enhanced by exogenously added HGF in a dose-dependent manner (Fig. 8). In contrast, HGF was not able to enhance the migration of control cell line NE1 (Fig. 8C).

Discussion

Previous studies have shown that HGF/SF-Met is involved in human malignancies (http://www.vai.org/metandcancer/). In several tumors, serum HGF levels were shown to be related to clinical outcome (23–25). Although a high concentration of HGF has been reported in ESCC tissue specimens (35), it remains unclear if serum HGF levels can be used as a biomarker. In the current study, we found that the serum HGF level was markedly elevated in patients with ESCC compared with control subjects. High serum HGF levels correlated with more advanced disease stage (tumor-node-metastasis stages III/IV). More importantly, we showed that serum HGF level was an independent prognostic factor on multivariate analysis. These results suggest that HGF may play an important role in the progression and tumorigenesis of ESCC.

HGF acts as a potent angiogenic molecule by directly acting on vascular endothelial cells. HGF stimulation of vascular endothelial cells promotes migration, proliferation, protease...
production, invasion, and organization into capillary-like tubes (36). Angiogenic factors IL-8 and VEGF, which promote tumor growth and metastasis (37–40), are often produced by tumor cells. Chen et al. (41) reported that serum levels of HGF were increased in parallel with VEGF and IL-8 in head and neck squamous cell carcinoma. In ESCC, increased expression of VEGF has been reported to correlate with tumor progression, poor treatment, and poor survival (3, 28). In the present study, we showed that serum levels of HGF, VEGF, and IL-8 were elevated in patients with ESCC. Levels of HGF correlated with that of IL-8 and VEGF, suggesting that the expressions of the three factors may be related. We further examined if HGF contributed in the regulation of angiogenic factor expression. Our data showed that HGF increased the secretion of VEGF and IL-8 in ESCC cells in a dose-dependent manner. HGF-induced IL-8 and VEGF expression was found to be dependent on ERK signaling pathways. The inhibition of ERK activation reduced HGF-induced IL-8 and VEGF expression, suggesting that this pathway is important in HGF-mediated production of angiogenic factors. These results provide a foundation for the hypothesis that HGF participates in angiogenesis via autocrine/paracrine mechanisms.

Overexpression of c-Met was reported by Saeki et al. (27) in ESCC tumor cells and cell lines. This was corroborated by our findings that ESCC cell lines and tumor cells in tissue specimens expressed c-Met. Furthermore, our study revealed that HGF was expressed by some of ESCC cell lines and immunohistochemical staining in tumor specimens showed that HGF was positive in tumor cells and stromal cells, such as fibroblasts, endothelial cells, and infiltrating cells. These results suggested that HGF and c-Met may constitute an autocrine/paracrine signaling system that plays a crucial role in the development of ESCC.

It has been reported that HGF could promote tumor cell migration in hepatocellular carcinoma (42) and other cancers (43, 44). Our in vitro migration assay provides further evidence that HGF enhances ESCC cell migration. HGF may play a role in promoting ESCC cell invasion and migration and tumor metastasis. In normal epithelial cells, however, HGF has no such effects. One possible explanation is the lack of c-Met on normal epithelial cell surface leading to unresponsiveness to HGF.

In conclusion, we have shown that serum HGF is increased in patients with ESCC. Serum HGF correlates with tumor stage and metastasis. Importantly, serum level of HGF is an
independent prognostic factor. Measurement of preoperative serum HGF levels may be useful and feasible in the clinical setting to predict metastasis and tumor stage and to guide treatment. It could also be hypothesized that HGF is a potential target to control angiogenesis and metastasis in ESCC. Cao et al. (45) reported that neutralizing HGF by antibodies can be a therapeutic strategy to regulate angiogenesis, growth, and metastasis of glioblastoma. In addition, it was shown that NK4, a truncated HGF that can bind to c-Met receptor without mediating biological responses, could serve as an antagonist to HGF and hence affects the motility and invasion of human colorectal cancer cells (46). Furthermore, geldanamycin at nanomolar concentrations has been shown to down-regulate c-Met expression and inhibit HGF-mediated cell motility and invasion in MDCK cells, the epithelial-derived cell line (47). These results suggest that neutralizing HGF production or inhibiting HGF/Met signaling may be used as potential therapeutic approaches in the management of ESCC. Further work is required to explore the role of HGF/Met and their use as therapeutic targets.

Significance of Serum HGF in Esophageal Cancer

References

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