Met Protein Expression Level Correlates with Survival in Patients with Late-stage Nasopharyngeal Carcinoma

Chao-Nan Qian, Xiang Guo, Brian Cao, Eric J. Kort, Chong-Chou Lee, Jindong Chen, Ling-Mei Wang, Wei-Yuan Mai, Hua-Qing Min, Ming-Huang Hong, George F. Vande Woude, James H. Resau, and Bin Tean Teh


ABSTRACT

Met tyrosine kinase, the receptor for HGF/SF, is important in various cellular functions, including proliferation, mitogenesis, formation of branching tubules, angiogenesis, and tumor cell invasion and metastasis. However, the role of Met/HGF signaling pathway in nasopharyngeal carcinoma (NPC) has not been evaluated. In this study, we determined the expression profile and clinical correlation of Met/HGF in 66 cases of NPC. Immunofluorescent staining and quantitative image analysis showed that the Met protein was expressed throughout the tumors and normal nasopharyngeal epithelia. Compared with NPC, the Met expression level was higher in columnar nasopharyngeal epithelium but lower in squamous nasopharyngeal epithelium. The normal interstitial stromal tissue expressed the lowest level of Met protein. HGF was detected mainly in the normal interstitial tissue surrounding the tumor. Met protein expression level was inversely correlated with patients’ survival time; the correlation coefficient was $-0.319 (P = 0.009)$. The mean survival time was 118 months in low Met expression group versus 52 months in high expression group ($P = 0.0004$). The cumulative 5-year survival rate was 77.68% in low expression group versus 38.24% in high expression group. The clinical stage was also significantly more advanced in high Met expression group. In the multivariate analysis, both clinical stage and Met protein expression level were independent prognostic indicators for patient survival. All of the five NPC cell lines tested did not express hgf mRNA but expressed met mRNA, and tyrosine phosphorylation of Met protein was mainly induced by exogenous HGF stimulation in these cells. No mutation was found in the tyrosine kinase and the juxtamembrane domains of Met receptor in the five NPC cell lines tested. These results indicate that: (a) high Met protein expression level correlates with poorer survival in late-stage NPC and serves as an independent prognostic indicator; and (b) the Met receptor in NPC is activated by its paracrine ligand HGF from the interstitial tissues rather than by an autocrine loop or its activating mutation.

INTRODUCTION

NPC$^2$ has its highest incidence rate in Southern China and is one of the most common cancers in Southeast Asia (1–3). The etiological factor is believed to be the interaction between genetic susceptibility and environmental factors, especially EBV (4, 5). The WHO developed a three-tier histological classification of NPC based on the degree of differentiation (6). These are squamous cell carcinoma, differentiated nonkeratinizing carcinoma, and undifferentiated carcinoma. In the endemic area, >95% of NPCs belong to the latter two types. But in nonendemic Western countries, squamous cell carcinoma account for 30–50% of NPC (7). Besides the epidemiological similarity, the serological and the clinical characteristics are also similar between differentiated nonkeratinizing carcinoma and undifferentiated carcinoma. In these two types of NPC, there is intense leukocyte infiltration, which predominantly comprises T lymphocytes and accessory cells (2, 8, 9). In addition to its rapid growth behavior, NPC has a great tendency to invade adjacent regions and metastasize to regional lymph node and distant organs. As a result, NPC is often locally advanced or already has spread to lymph nodes at time of diagnosis. Together these factors account for the high rate of treatment failure despite the tumor cells’ radiosensitivity (1, 10, 11).

The Met tyrosine kinase receptor, whose ligand is HGF/SF (12), was identified originally as a transforming gene activated by rearrangement in cells treated with a chemical carcinogen (13, 14). The MET protooncogene contains 21 exons which give rise to four domains (15, 16); the extracellular domain (exons 2–13), the transmembrane domain (exon 13), the juxtamembrane domain (exon 14), and the tyrosine kinase domain (exons 15–21). Missense mutations in the tyrosine kinase domain and in the juxtamembrane domain have been found to be tumorigenic and able to induce metastasis by constitutively activating the Met tyrosine kinase activity (17–24).

The Met protein is a tyrosine kinase receptor synthesized as a $M_r$ ~170,000 precursor that is glycosylated and cleaved to create the mature protein (25, 26). As a cell membrane receptor, the Met protein is composed of two subunits: an extracellular $\alpha$ chain of $M_r$ ~50,000 and a transmembrane $\beta$ chain of $M_r$ ~145,000 (25). There is a single multifunctional docking site located in the tail of the $\beta$ chain. Its ligand, HGF/SF, is a heterodimeric glycoprotein composed of a $M_r$ ~69,000 $\alpha$ chain and a $M_r$ ~34,000 $\beta$ chain. After the stimulation of HGF, the docking site is phosphorylated on two adjacent tyrosine residues leading to the recruitment and activation of multiple transducers and signaling pathways involving proliferation, mitogenesis, formation of branching tubules, angiogenesis, and tumor cell invasion and metastasis (27, 28). Although Met and HGF/SF are expressed predominantly in cells of epithelial and mesenchymal origin, respectively (29, 30), Met overexpression has been reported in a variety of cancers and cell lines from the tissues of breast (31, 32), ovary (33), thyroid (34), pancreas (35), stomach (36), brain (37), prostate (38), and endometrium (39). However, its expression profile in NPC has not been studied.

In the present study, we investigated the expression profiles of Met/HGF in NPC, the clinical correlation of Met with advanced NPC, and the activation mechanism of Met receptor in NPC cell lines.

MATERIALS AND METHODS

Patients and Tissue Samples. A total of 66 patients with histologically, clinically, and radiographically diagnosed late-stage NPC from the Cancer Center, Sun Yat-sen University of Medical Sciences, between December 1982 and December 1996 was characterized. There were 48 males and 18 females with an average age of 46.4 years (range, 20–70 years). The WHO histological classification system was used for pathological stratification. Standard curative...
radiotherapy was applied to the patients with local or locoregional diseases. Platinum-based concomitant and/or adjuvant chemotherapies combined with alleviatory radiotherapy were used for the patients with distant dissemination NPC. The median follow-up time was 79 months (range, 1–163 months). Survival time was calculated from the date of treatment until the time of death or the most recent follow-up if the patient was alive. At the end of the study period (December 1996), 37 patients (56%) had died as a result of recurrence of their disease. The patients and tumor-related characteristics are shown in Table 1. The formalin-fixed, paraffin-embedded archived primary tumor biopsy tissues of all patients before treatment were retrieved and used in the analyses. Consecutive 4-μm paraffin sections were prepared for all tissues. One set of the sections was used for routine H&E staining and were reviewed and reclassified using WHO histological classification. The remaining sections were used for further immunofluorescent staining.

**Clinical Staging System.** The disease stages of all patients were classified or reclassified according to the 1992 NPC staging system of China (40). The staging system is characterized according to the following model: T, primary tumor; T1, limited to the nasopharynx; T2, involvement of the nasal cavity, oropharynx, soft palate, anterior cervical vertebrae soft tissue, and parapharyngeal space extension before the SO line (the SO line is between the styloid process and the midpoint on the posterior edge of the great occipital foramen); T3, extension over the SO line, involvement of the anterior or posterior cranial nerves, parabasal sinus, cavernous sinus, orbit, infratemporal fossa, and direct invasion of the palatine fossa; T4, involvement of both anterior and posterior cranial nerves, nerves alone, the base of the skull, the pterygoprocess zone, and the pterygoal canal.

**Distant metastasis:** M0, absence of distant metastasis; M1, presence of distant metastasis; M1a, M1b.

**Histologic type:** WHO type, undifferentiated carcinoma; WHO type, differentiated nonkeratinizing carcinoma.

**Cell Culture, HGF, and Antibodies.** In addition to the primary biopsies, two well-differentiated NPC cell lines, CNE-1 and HK-1, were used, as well as three poorly differentiated NPC cell lines: CNE-2, HONE-1, and SUNE-1 (41–45). S-114 is a cell line transformed from NIH 3T3 cells that stably coexpresses human Met and HGF, resulting in autophosphorylation of Met receptor (46, 47). We used this cell line for positive control in Northern blot analyses. The five NPC cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum, whereas S-114 was cultured in DMEM, supplemented with 8% calf serum. Penicillin (100 IU/ml) and 100 μg/ml streptomycin were added to the culture media.

Human recombinant HGF/SF was purified from the supernatant of transformed NIH 3T3 cells as described previously (47). HGF/SF concentrations were presented as scatter units per ml; five scatter units are equivalent to ~1 ng of protein.

The anti-hHGF monoclonal antibody was prepared in the Lab of Monoclonal Antibody Production, VARI (46). For immunofluorescent studies, the anti-hHGF monoclonal antibody was used at 20 μg/ml in blocking buffer (10% BSA and 5% donkey serum in PBS); the polyclonal rabbit anti-hMet antibody (C-28; Santa Cruz Biotechnology, Santa Cruz, CA.) was used at 4 μg/ml blocking buffer. Negative control antibodies were nonspecific mouse IgG1, (R&D Systems, Minneapolis, MN; catalogue no. MAB004) and normal rabbit IgG (R&D Systems; catalogue no. AB-105-C) used at 10 μg/ml. Secondary antibodies were 8 μg/ml rhodamine-conjugated donkey antiserum immunoglobulins and 12 μg/ml FITC-conjugated donkey antirabbit immunoglobulins (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in blocking buffer. For immunoprecipitation, mouse monoclonal anti-human Met antibody (Upstate Biotechnology, Lake Placid, NY; Info-ID 05237) was used as 0.5 μl/sample. For Western blot analysis, mouse monoclonal antihuman Met antibody (Upstate Biotechnology; Info-ID 05238) was used in the dilution of 1:2,500, mouse monoclonal antiphosphotyrosine antibody (Info-ID 05321) was used in the dilution of 1:1,000, and goat antirabbit horse-radish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) was diluted to 1:10,000.

**Immunofluorescent Double Staining.** The sections for immunofluorescent double staining were deparaffinized, rehydrated, and then boiled in 10 mm sodium citrate buffer (pH 6.0) for 10 min to increase antigen exposure. Nonspecific antibody-binding sites in the tissue sections were blocked by incubation in blocking buffer for 30 min at room temperature. The sections were incubated overnight at 4°C with both mouse anti-hHGF and rabbit anti-hMet antibodies. For negative control staining, nonspecific mouse IgG and rabbit IgG were used in place of the primary antibodies. The sections were then washed three times with PBS containing 0.5% Tween 20 to move unbound primary antibodies followed by incubation in fluorochrome-conjugated secondary antibodies for 30 min at room temperature. The sections were finally rinsed with PBS containing 0.5% Tween 20 three times to remove unbound secondary antibodies and mounted in Gel/Mount permanent aqueous mounting medium (Biomeda Corp, Foster City, CA).

**Quantitative Fluorescent Analyses.** The techniques and methods of quantitative fluorescent analyses have been described previously (21–23, 48). Briefly, a Zeiss LSM410 confocal laser scanning microscope configured with a 25-mW argon internal HeNe laser was used to image the fluorescent-stained sections with lines 488 and 543 for FITC and rhodamine excitation, respectively. The 635 red laser was used to produce Nomarski images. The fluorescent intensity was captured via the microscope’s photomultiplier tube as a 512 × 512 pixel image file with an image depth of 8 bits (i.e., each pixel was stored as an intensity ranging from 0 to 255). The images were quantified from the digital microscope images using software developed by Laboratory of Analytical, Cellular, and Molecular Microscopy, VARI. Analysis of each sample was made with the same laser intensity, brightness, power, and scan rate. The regions of interest in each image were defined using Paint Shop Pro software version 5.01 (Jasc Software, Minneapolis, MN). The average fluorescence intensity of each region of interest was calculated using Image Pro Plus software. The area with intensity <20 was excluded because that corresponded to only glass or paraffin without any tissue. For each tissue, two to three images were obtained, and the average value was used as the detection value.

**Northern Blot Analysis.** The cDNA probe for human hfg was derived from the coding region for HGF α chain (accession no. X16323) by PCR amplification using the primer pairs: forward primer, 5’-TGCTGGTGAC-CAAAAGCTTGGCCG-3’ (nucleotides 136–167) and reverse primer, 5’-TCG-
CAGTTGTTCTGTTCGTCG-3’ (nucleotides 1596–1616). The cDNA probe for human met was derived from the coding region containing exons 15 and 16 (accession no. X54559.1) by PCR amplification using the primer pairs: forward primer, 5’-ATCTCAGAAACGGTCTAGC-3’ (nucleotides 3239–3258) and reverse primer, 5’-CATCTAGGTCACCAAGTGTC-3’ (nucleotides 3479–3498). PCR products were then separated on a 0.8% agarose gel and purified using Qiagen II gel extraction kit (Qiagen, Inc., Valencia, CA; catalogue no. 20021). Human β-actin cDNA was purchased from Ambion, Inc. (Austin, TX; catalogue no. 7424–10 μl). Total cellular RNA was prepared by a rapid extraction method using the TRIzol reagent according to the manufacturer’s instructions (Life Technologies, Inc.) and subjected to electrophoresis through 1% formaldehyde-agarose gels followed by transfer to Hybond-XL nylon membranes (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, England). Blots were prehybridized and hybridized at 65°C using Rapid-Hyb buffer (Amersham Pharmacia Biotech UK Ltd.) according to the manufacturer’s instructions. The cDNA probes were labeled using the random prime labeling system Rediprime II (Amersham Pharmacia Biotech UK Ltd.). Hybridization occurred in the present of [α-32P]dCTP-labeled cDNA probes for hgf, met, or β-actin. The membrane was washed at room temperature with 2 × SSC (150 mM sodium chloride and 15 mM sodium citrate), 0.05% SDS for 40 min, and at 50°C in 0.1 × SSC, 0.1% SDS for 30 min. And then the membrane was exposed to Kodak X-Omat film using intensifying screens at −80°C for 4–72 h.

Immunoprecipitation and Western Blot Analysis. The cells with 80% confluence were starved with serum-free DMEM supplemented by 0.1% BSA for 12 h. Human recombinant HGF was then added at the concentration of 100 unit/ml 10 min before harvest. The cells were washed twice with ice-cold PBS and then lysed on ice for 20 min in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% Na-deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM sodium vanadate. The insoluble debris was removed by centrifugation at 14,000 × g for 15 min at 4°C. A total of 300 μg/300 μl lysate from each cell line was incubated with 0.5 μl of antihuman Met antibody overnight in 4°C, and the immunocomplexes were precipitated with protein G-Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The immunoprecipitated proteins were washed three times with lysis buffer and separated using precast 10% Tris-glycine acrylamide gels (Introgen and Living Science; EC60752). The proteins were electrophoretically transferred from the gels to nitrocellulose membrane. The membranes were blocked with 3% nonfat dry milk in TTBS [10 mM Tris-HCl (pH 8.4), 150 mM sodium chloride, and 0.05% Tween 20] for 1 h at room temperature and then incubated with primary antibodies (anti-hMet or antiphosphotyrosine) diluted in 10 mM of TTBS–BSA (TTBS with 2% BSA) for 1 h at room temperature. After being washed with TTBS, the membranes were incubated with secondary antibody diluted in TTBS–BSA and again washed with TTBS. An enhanced chemiluminescence kit (Fierce) was used to detect the protein. The membranes were finally exposed to films from 30 s to 3 min.

MET Mutation Screening in NPC Cell Lines. All of the five NPC cell lines were collected for mutation screening in exons 14 and 16–21 of MET gene. The genomic DNA was prepared by using the TRIzol reagent according to the manufacturer’s instructions. Sequencing primers for these exons are listed in Table 2. PCR was performed in a 50-μl reaction volume containing 50 ng of DNA; 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 1.5 mM MgCl2; 0.2 μM each primer; 0.2 mM dATP, dGTP, dCTP, and dTTP; and 2 μl of each cDNA by means of a programmable thermal cycler (GeneAmp PCR system 9700; Perkin-Elmer) at the following settings: after a denaturation at 94°C for 5 min, samples were amplified for 35 cycles at 94°C, 30 s; 55°C–58°C, 30 s; and 72°C, 45 s, with a final extension at 72°C for 10 min. After amplification, all of the PCR products were subjected to purification using Microcon YM-100 column (Amicon and Millipore) following the manufacturer’s manual. Purified PCR products were subjected to direct DNA sequencing using ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems).

Statistical Analysis. Mann-Whitney U test was used to compare the means of fluorescent intensity among different tissues. Spearman’s rank-sum correlation coefficients were calculated to study the correlation between patient survival time and fluorescent intensity of Met protein. Estimates of cumulative survival were calculated using Kaplan-Meier method. Observed differences in survival were analyzed by Log-rank test. Multivariate analysis was performed by Cox’s proportional hazards regression model. Student’s t test was used to compare the difference of age between the two groups. To test the association between Met expression level and other clinicopathological characteristics, Fisher’s exact test was performed. All tests were two tailed. A P < 0.05 was defined as statistically significant.

RESULTS

Expressions of Met and HGF Proteins in NPC Biopsy Tissues. With either the H&E-stained images alone or the Nomarski and H&E combinations, the tumor tissues, non-tumor columnar and squamous epithelial tissues, and normal nonepithelial tissues could be easily identified by standard histopathology criteria. Among these 66 primary tumor tissues, 12 of them contained normal, adjacent, and uninvolved nasopharyngeal columnar epithelia, 6 cases contained uninvolved nasopharyngeal squamous epithelia, and 55 cases contained normal nonepithelial interstitial tissues surrounding the tumors. Specific fluorescent staining was obtained in all 66 cases. Met protein was expressed throughout the cytoplasm of most of the tumors cells and epithelial cells (Figs. 1 and 2). Fluorescent intensity was detected and evaluated in four kinds of tissues: primary tumor, columnar nasopharyngeal epithelium, squamous nasopharyngeal epithelium, and normal nonepithelial interstitial tissue. The results showed that the highest expression level of Met protein was in nasopharyngeal columnar epithelium, followed by NPC. However, the Met intensity level in NPC was significantly higher than squamous epithelium and normal interstitial tissue. The marginal significance of the difference in Met expression level between squamous epithelium and interstitial tissue may be less cases of squamous epithelial tissue were studied. The Met protein expression levels among these four kinds of tissues were shown in Fig. 3. HGF/SF was detected mainly in the interstitial tissues surrounding the tumor (Fig. 1). Only scattered spots in the tumor tissues were stained with HGF/SF antibodies. The expression pattern of Met/HGF in normal, adjacent nasopharyngeal columnar epithelium was different compared with that of normal squamous epithelium. As shown in Fig. 1, Met expression level was lower in the tumor cells compared with the expression level in the columnar epithelial cells, whereas it was higher in the neoplastic cells compared with squamous epithelium in Fig. 2.

The Correlation of Met Protein Expression with Clinical Parameters. The expression level of Met protein in NPC was significantly correlated with patients’ survival time (P = 0.009); the correlation coefficient was −0.319, indicating that higher levels of Met expression was correlated with shorter survival time. A 4-fold table method (49) was used to calculate the sensitivity and specificity of

<table>
<thead>
<tr>
<th>Exons</th>
<th>Forward primers (5′-3′)</th>
<th>Reverse primers (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>14</td>
<td>CCATGATAGCGCCTTTA</td>
<td>CAATGTCAGAACCCACTGT</td>
</tr>
<tr>
<td>16</td>
<td>ATTATCTCTGCAAGCTCTAAC</td>
<td>GGTTGCAAACCAAAAGATAT</td>
</tr>
<tr>
<td>17</td>
<td>GATTTGGTGTCTACATAAGAAGT</td>
<td>GATGCTCGGTCTACAGCTATT</td>
</tr>
<tr>
<td>18</td>
<td>AACAGTATGCTCTTATCTGTT</td>
<td>AACAGATCCCTCCTGACCCTT</td>
</tr>
<tr>
<td>19</td>
<td>TCTTATCTGACACGGTTAAT</td>
<td>ATGAAAGTAAAGGAGGGAATCT</td>
</tr>
<tr>
<td>20</td>
<td>TATGGTATGCTACATCTCAC</td>
<td>CTTTTGAGGAGGCACTTTCTG</td>
</tr>
<tr>
<td>21</td>
<td>ACAGAATTGCTGCTTCAAAAGG</td>
<td>CGGAGCCACAATTTACGTCAC</td>
</tr>
</tbody>
</table>

Table 2 Primer sequences for MET mutation screening
each cutoff point of Met staining intensity in predicting patients’ survival time, which was defined as short-term survival if the survival time was ≤36 months or long-term survival if the survival time was >36 months. The cutoff value of 94 was chosen to distinguish high and low expression, as the largest product of sensitivity multiplied by specificity was obtained at this level. Table 3 showed the patient classification using the cutoff value of 94 and survival time. The sensitivity and specificity of Met expression in predicting survival time were 72 and 65%, respectively, in this classification. Separation of the patients into low Met expression group (n = 32) and high Met expression group (n = 34) under this cutoff point was shown in Table 1. Kaplan-Meier survival analysis showed that in low Met expression groups, the mean survival time was 118 months, 95% CI, 95–141. However, the mean survival time in high expression group was only 52 months, 95% CI, 35–69 (Fig. 4). Log-rank test showed that the survival time was significantly different between these two groups (P = 0.0004); the low Met expression group had better survival, whereas the high Met expression group had worse survival. The cumulative 5-year survival rate was 77.68% in low expression group, but it was only 38.24% in high expression group. The clinical stage was significantly later in high Met expression group (P = 0.005). No significant difference had been found in age, gender, histological stratification, T stage, N stage, or M stage between the two Met expression groups. Besides Met expression level, N stage, M stage, and clinical stage were also significantly correlated with survival in Kaplan-Meier analysis and Log-rank test (for N stage, P = 0.015; for M stage and clinical stage, P < 0.001). Therefore, the initial multivariate model included Met expression level, N stage, M stage, and clinical stage (to control for the possible effect of covariance between those terms with significant correlation coefficients). No interaction terms were significant, except for that of M stage and clinical stage, which was incalculable because of their complete congruence. Therefore, M stage was removed from the final model along with its interaction terms. The remaining interaction terms and the N stage were removed from the model using the stepwise backwards elimination method and a retention threshold of
The resulting final model was shown in Table 4, indicating that the clinical staging and Met protein expression level were independent prognostic indicators for survival.

Expression of Met and HGF mRNA in NPC Cell Lines. To further clarify the expression profiles of met and hgf in NPC, five NPC cell lines were used for the mRNA evaluation. All of the five NPC cell lines expressed met mRNA but did not express hgf in Northern blot analysis (Fig. 5). These results were consistent with the finding in the immunofluorescent staining study of primary tumors, implying that an autocrine HGF-Met loop was not involved in the activation of the Met receptor in NPC.

Response of Met Receptor to HGF Stimulation. The paracrine activation mechanism of Met receptor in NPC was verified by exogenous stimulation of HGF in NPC cells. In all of the five NPC cell lines, Met tyrosine phosphorylation could be induced by exogenous HGF stimulation (Fig. 6). Although some extent of autophosphorylation of Met protein occurred in CNE-2, it also responded to HGF as more phosphorylated Met protein was detected after the HGF stimulation.

Table 3 The 4-fold table data classified according to patients with short-term and long-term survival and met expression levels

<table>
<thead>
<tr>
<th>Met expression (fluorescent intensity)</th>
<th>Short-term survival (patient no.)</th>
<th>Long-term survival (patient no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;94</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>≤94</td>
<td>8</td>
<td>24</td>
</tr>
</tbody>
</table>

Fig. 4. Kaplan-Meier survival analysis showed that the cumulative 5-year survival rate was 77.68% in the low Met protein expression group (n = 32), but it was only 38.24% in the high expression group (n = 34). The mean survival time in the low Met protein expression group was 118 months (95% CI, 95–141), but it was only 52 months (95% CI, 35–69) in the high expression group.

Response of Met Receptor to HGF Stimulation. The paracrine activation mechanism of Met receptor in NPC was verified by exogenous stimulation of HGF in NPC cells. In all of the five NPC cell lines, Met tyrosine phosphorylation could be induced by exogenous HGF stimulation (Fig. 6). Although some extent of autophosphorylation of Met protein occurred in CNE-2, it also responded to HGF as more phosphorylated Met protein was detected after the HGF stimulation.
Mutation Detection in MET Oncogene. The possibility of activating mutation in MET oncogene was investigated by sequencing the six exons of MET gene coding for juxtamembrane and tyrosine kinase domains of the protein. No mutation of MET oncogene was found in all of the five NPC cell lines. However, polymorphisms were found in exons 20 and 21 in HONE-1. Therefore, we screened exons 20 and 21 for MET mutation in five NPC cell lines. We did not find any mutation in these exons; only polymorphisms were found in exons 20 and 21 in HONE-1. Therefore, mutation in the exons of MET oncogene coding for the juxtamembrane and the tyrosine kinase domains is not involved in the activation of Met receptor in NPC. Interestingly, we found some extent of autophosphorylation of Met receptor in one NPC cell line: CNE-2. The mechanism of this constitutive activation is unclear. The possibility of the existence of novel activation mechanism(s) in this cell line is worthwhile for further investigation.

The role of HGF/SF in many tumors is as a stroma-derived factor that promotes invasion and metastasis of cancer cells (53–55). But in some cancers, an autocrine HGF-Met loop is involved (56, 57). In the present study, HGF/SF was found to be expressed neither in NPC tissues nor in NPC cell lines. However, HGF/SF was abundantly detected in the interstitial tissues surrounding NPC, in which lymphocyte has been reported to be the predominant cell type (2). The strong phosphorylation response of Met protein in NPC cells to the exogenous HGF stimulation, which itself is an indicator of activation of multiple signal transduction pathways, implied that HGF acted as a paracrine factor in NPC development. The influence of the interstitial tissue, especially the lymphocytic infiltration in NPC tissues, is controversial. Although correlation of rapid tumor development and distant metastasis with reduced lymphocyte infiltration has been reported (8), others found that lymphocytic infiltration was not statistically related to a better survival. (9) But a higher density of dendritic cells and monocytes/macrophages in NPC tissues has been shown to correlate with good prognosis, fewer lymph node metastases, and fewer distant metastases (8, 9). Further study should clarify the specific cell type that is secreting HGF/SF and its impact on the promotion and development of NPC.

The tissue origin of NPC is not very clear. Although NPC is mostly found in the regions covered by columnar nasopharyngeal epithelium, it possesses certain characteristics of both columnar and squamous epithelia (58, 59). Moreover, it has been reported that NPC can be multifocal in origin (60). Our results showed that the expression level of Met in NPC was lower than in columnar nasopharyngeal epithelia but higher than in squamous epithelia. The variance in the expression level of Met protein among normal epithelial tissues and NPC tissue circulating VEGF (51) and EBV DNA (52), may improve both the sensitivity and specificity in predicting individual patient’s prognosis. Mutations in MET oncogene have been found to be tumorigenic and able to induce metastasis by constitutively activating the Met tyrosine kinase activity. Missense mutations in exons 16–21 coding for the tyrosine kinase domain were discovered in hereditary and sporadic human papillary renal carcinoma, ovarian cancer, and hepatocellular carcinoma (17–23). Another missense mutation in exon 14 coding for the juxtamembrane domain with a regulatory site was reported recently in primary gastric adenocarcinoma (24). In the present study, we screened exons 14 and 16–21 for MET mutation in five NPC cell lines. We did not find any mutation in these exons; only polymorphisms were found in exons 20 and 21 in HONE-1. Therefore, mutation in the exons of MET oncogene coding for the juxtamembrane and the tyrosine kinase domains is not involved in the activation of Met receptor in NPC.

**DISCUSSION**

Although NPC has remarkable radiosensitivity and chemosensitivity, the 5-year survival rate in late-stage NPC patients is still ~30%, especially in patients with bulky regional lymph node involvement (1, 40). Distant metastasis of NPC is more frequent than most of other head and neck cancers (1), and it remains the most common reason for treatment failure despite improvement in the treatment modality in recent years by combining radiotherapy with chemotherapy (50). Efforts to find any effective biomarker to predict the prognosis of the patients with late-stage NPC are therefore warrantied to improve the cure rate. In the present study, we explored for the first time the correlation between Met protein expression and survival time in 66 cases of late-stage NPC investigated. We found that Met protein expression was inversely correlated with survival time. The patients with higher Met protein expression had shorter survival time. The Met protein expression level was also significantly correlated with the clinical staging, with more cases of stages IVA and IVb in high Met expression group (Table 1). Met expression was not correlated with primary tumor development in our study. Its correlations with the N and M staging also did not reach statistical significance, which may be explained by the reason that the sample size might not be large enough to reach statistical significance, especially in the M staging, because only five cases with distant metastasis were studied. Moreover, both Met protein expression level and clinical staging were independent predictors in multivariate analysis. Prospective clinical study is necessary to confirm that Met protein is one of the reliable clinical predictors of outcome for individual patients with late-stage NPC. It is likely that combination of Met with other biomarkers, such as

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**Table 4 Analysis of 66 cases of NPC by Cox’s proportional hazards regression model**

<table>
<thead>
<tr>
<th>Factors</th>
<th>β</th>
<th>SE</th>
<th>P</th>
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<tbody>
<tr>
<td>Clinical staging</td>
<td>1.3224</td>
<td>0.3443</td>
<td>0.0001</td>
</tr>
<tr>
<td>Met expression level</td>
<td>0.7936</td>
<td>0.3926</td>
<td>0.0432</td>
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**Fig. 5. Northern blot analysis was performed to evaluate the mRNA expressions of hgf (A) and met (B) in five NPC cell lines compared with positive control cell line S-114. β-actin expression was probed to monitor the equal mRNA sample loading.**

**Fig. 6. Phosphorylation of Met receptor in NPC cell lines under the stimulation of HGF.** The cells were starved under serum-free media overnight before HGF was added to the media. Then the proteins were extracted for immunoprecipitation (IP) using anti-hMet antibody. The precipitated proteins were then assayed by Western blot (WB) analyses using antiphosphotyrosine (anti-P-tyr) antibody and anti-hMet antibody.
may be in part attributable to different transcription activity. The exploration to clarify the mechanism that shifts Met/HGF signaling pathway from differentiation in normal epithelium to the proliferation and the invasion in malignant cells would undoubtedly be helpful to fully understand the role of Met/HGF in NPC development.

Angiogenesis is a critical process in the development and metastasis of many solid tumors, including NPC (51, 61). We found previously that serum VEGF level was elevated in the patients with metastatic NPC (50), and vasculature density in primary NPC was correlated with distant metastasis (61). In vivo experiments showed that antiangiogenic agents could not only suppress the tumor growth of NPC cell lines (62, 63) but also enhance the therapeutic effect of the conventional cytotoxic drug 5-fluorouracil, which is one of the first line drugs in chemotherapy of NPC (64). HGF/SF is a well-known up-regulator of angiogenesis via activating the Met/HGF signaling pathway (65). HGF was reported to act in concert with VEGF to promote human endothelial cell survival and tubulogenesis (66). Furthermore, in a recent study, HGF was found to be able to promote expression of angiogenesis factors interleukin-8 and VEGF in head and neck cancer cell lines through both mitogen-activated protein/ extracellular signal-regulated kinase kinase- and phosphatidylinositol 3'-kinase-dependent pathways (67). It will be worthwhile to whether blocking the Met/HGF pathway would benefit the tumor control in NPC patients via inhibiting angiogenesis in addition to inhibiting tumor invasion and proliferation.

In summary, this study demonstrates that the expression level of Met protein in NPC stained immunofluorescently and evaluated quantitatively by confocal microscopy is inversely correlated with the survival time and directly correlated with clinical stage of patients with late-stage NPC. Both clinical staging and Met expression level are independent prognostic factors in the multivariate analysis. HGF acted as a paracrine factor in the activation of Met receptor in NPC cells. Met protein may be a potential biomarker to predict poor prognosis in NPC patients, and blocking Met/HGF signaling pathway may be beneficial in the treatment of NPC.

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Note Added in Proof

After submission of the present manuscript, Horikawa et al. (68) published their work on October 29, 2017. © 2002 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from
Met Protein Expression Level Correlates with Survival in Patients with Late-stage Nasopharyngeal Carcinoma

Chao-Nan Qian, Xiang Guo, Brian Cao, et al.