Presence of Phosphorylated Hepatocyte Growth Factor Receptor/c-Met Is Associated with Tumor Progression and Survival in Patients with Conventional Renal Cell Carcinoma

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Abstract

Purpose: Hepatocyte growth factor receptor (HGFR/c-Met) signaling is associated with tumor progression in various cancers. The clinical significance and pathologic roles of phosphorylated HGFR/c-Met in renal cell carcinoma (RCC) are not fully understood; therefore, this study sought to clarify the possible role of two tyrosine residues (pY1234/pY1235 and pY1349) in HGFR/c-Met.

Experimental Design: The kinetics of tyrosine phosphorylation at these two residues was examined in a human renal carcinoma cell line, ACHN cells. In addition, phosphorylated HGFR/c-Met expression (using phosphorylation site-specific antibodies for pY1234/pY1235 and pY1349) was examined in 114 tumor sections of conventional RCC patients by immunohistochemistry. The relationships between these expressions and clinicopathologic features and survival were also investigated.

Results: Although phosphorylation of Y1349 HGFR/c-Met was observed for 120 minutes after HGF treatment of ACHN cells, maximal phosphorylation of Y1234/Y1235 was observed at 30 minutes followed by a rapid inactivation. Median rates (range) of cancer cells immunopositive for pY1234/pY1235 HGFR/c-Met and pY1349 HGFR/c-Met in the tumor sections were 0% (0-5.2%) and 14.3% (0-64.3%), respectively. Positive expression of pY1349 HGFR/c-Met was significantly associated with high pT stage, presence of metastasis, and high-grade carcinoma. Multivariate Cox analysis revealed that the positive expression of pY1349 HGFR/c-Met was a significant and an independent predictor of cause-specific survival (odds ratio, 2.94; 95% confidence interval, 1.12-7.72; $P = 0.028$).

Conclusions: Phosphorylated HGFR/c-Met may be important in the tumor progression of RCC. Expression of pY1349 HGFR/c-Met is a useful predictor for metastasis and survival of conventional RCC patients.

Hepatocyte growth factor receptor (HGFR)/c-Met belongs to the tyrosine kinase receptor family of proteins and signals via HGFR/c-Met in a variety of normal cellular processes (1, 2). In addition to normal physiologic functions, HGFR/c-Met signaling is also implicated in oncogenic processes and malignant aggressiveness, such as tumor growth, invasion, and metastasis (3–6). Down-regulation of HGFR/c-Met by small interfering RNA or inhibition of its receptor tyrosine kinase by small-molecule inhibitors significantly inhibited proliferation and survival of tumor cells *in vitro* as well as tumor growth (7–10). Furthermore, increased cellular levels of HGFR/c-Met have been correlated with tumor progression and patient outcome in various cancers (11–13).

Renal cell carcinoma (RCC) is a heterogeneous disease with several distinct genetic backgrounds (14). Most of these tumors can be classified as conventional RCC and are frequently associated with mutations of the *VHL* tumor suppressor gene. Activating mutations of HGFR/c-Met have been reported in hereditary type 1 papillary carcinoma (4). Although the same mutation is uncommon in sporadic papillary RCC and is not a typical feature of sporadic conventional RCC, overexpression of HGFR/c-Met is frequently observed (15, 16) and it causes ligand-independent activation of receptor tyrosine kinases (17, 18). Downstream of overexpressed HGFR/c-Met, phosphoinositide 3-kinase activity is increased (18), which may stimulate proliferation, migration, and survival of renal carcinoma cells through c-Akt and p70 S6 kinase (5, 6). Thus, activated HGFR/c-Met may affect the progression of common RCC as well.

Although mutations and overexpression of HGFR/c-Met have been extensively studied in human malignant tumors, the significance of phosphorylated HGFR/c-Met in malignancies is relatively unknown. Phosphorylated HGFR/c-Met expression was detected in gastric cancers and non–small cell lung cancers (19, 20). However, the direct correlation between

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Received 2/15/06; revised 5/19/06; accepted 6/8/06.

Grant support: Japan Society for the Promotion of Science for the Grant-in-Aid for Japan Society for the Promotion of Science (B) (2) 16390467 (H. Kanetake).

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doi:10.1158/1078-0432.CCR-06-0362

Table 1. Clinicopathological features and their relationship to HGFR/c-Met status

Variables	No. patients	HGFR/c-Met expression		pY1349 HGFR/c-Met	
		Negative	Positive	Negative	Positive
No. patients	114	41	73	68	46
Gender					
Male	86	35/85.4	51/69.9	55/80.9	31/67.4
Female	28	6/14.6	22/30.1	13/19.1	15/32.6
P		0.065		0.101	
Age (y)					
Median or less (≤61)	57	16/52.4	41/51.1	33/48.5	24/52.2
Over median (>61)	57	25/47.6	32/48.9	35/51.5	22/47.8
P		0.079		0.703	
pT stage					
pT1	69	30/73.2	39/53.4	51/75.0	18/39.1
pT2	17	5/12.2	12/16.4	9/13.2	8/17.4
pT3	24	6/14.6	18/24.7	8/11.8	16/34.8
pT4	4	0/0.0	4/5.5	0/0.0	4/8.7
P		0.138		<0.001	
Lymph node metastasis					
Absence	105	39/95.1	66/90.4	66/97.1	39/84.8
Presence	9	2/4.9	7/9.6	2/2.9	7/15.2
P		0.247		0.014	
Distant metastasis					
Absence	97	37/90.2	60/82.2	62/91.2	35/76.1
Presence	17	4/9.8	13/17.8	6/8.8	11/23.9
P		0.247		0.027	
Grade					
Grade 1	41	18/43.9	23/31.5	30/44.1	11/23.9
Grade 2	57	20/48.8	37/50.7	34/50.0	23/50.0
Grade 3 + 4	16	3/7.3	13/17.8	4/5.9	12/26.1
P		0.202		0.004	

phosphorylated HGFR/c-Met expression and clinicopathologic features in human cancer has yet to be elucidated, particularly for RCC.

In the present study, we show for the first time a significant association between tumor growth and progression of conventional RCC and the expression of phosphorylated HGFR/c-Met affecting tumor size, invasion, cancer cell proliferation, and metastasis. Our results also showed that phosphorylated HGFR/c-Met was directly linked with malignant aggressiveness.

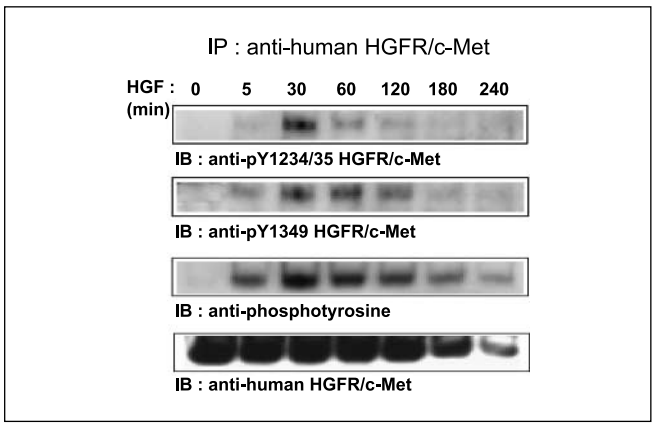

Fig. 1. Kinetics of tyrosine phosphorylation of HGFR/c-Met in a human renal carcinoma cell line, ACHN cells. ACHN cells grown in 6-cm dishes were serum starved overnight. Cells were then left unstimulated or stimulated with 100 ng/mL HGF for indicated periods. HGFR/c-Met was immunoprecipitated (*IP*), separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and immunoblotted (*IB*) with indicated antibodies. Reproducible data were obtained from two independent experiments.

In addition, multivariate analysis showed that positive expression of tumor tissue for the phosphorylated receptor was a strong predictor of cause-specific survival. Together, our results strongly implicate phosphorylated HGFR/c-Met in tumor development and prognosis in patients with conventional RCC.

Materials and Methods

Patients and tumor samples. Data from patients pathologically diagnosed with conventional RCC were reviewed retrospectively. Patients with a history of malignancy were excluded from this study, as were patients who had previously received neoadjuvant therapy. All patients were treated with radical nephrectomy, and tumors were staged according to the 1997 tumor-node-metastasis staging system. Nuclear grading was based on the criteria of Fuhrman et al. (21). In the present study, tumors were grouped for statistical analysis into the following groups: low stage (pT1 and pT2) and high stage (pT3 and pT4) or low grade (grades 1 and 2) and high grade (grades 3 and 4). We also studied the normal tissues adjacent to the tumor as a control. Tumor size was examined histopathologically for resected specimens, and maximum size was used for statistical analyses. The study protocol met the ethical standards of the Human Ethics Review Committee of Nagasaki University School of Medicine (Nagasaki, Japan).

Antibodies. Anti-human HGFR/c-Met for immunostaining was obtained from Zymed Laboratories, Inc. (South San Francisco, CA). Anti-human HGFR/c-Met for immunoprecipitation and immunoblotting and an anti-phosphotyrosine (pY) monoclonal antibody (PY99) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-specific antibodies against human HGFR/c-Met (pY1234/pY1235 HGFR/c-Met and pY1349 HGFR/c-Met) were purchased from Cell Signaling Technology (Beverly, MA).

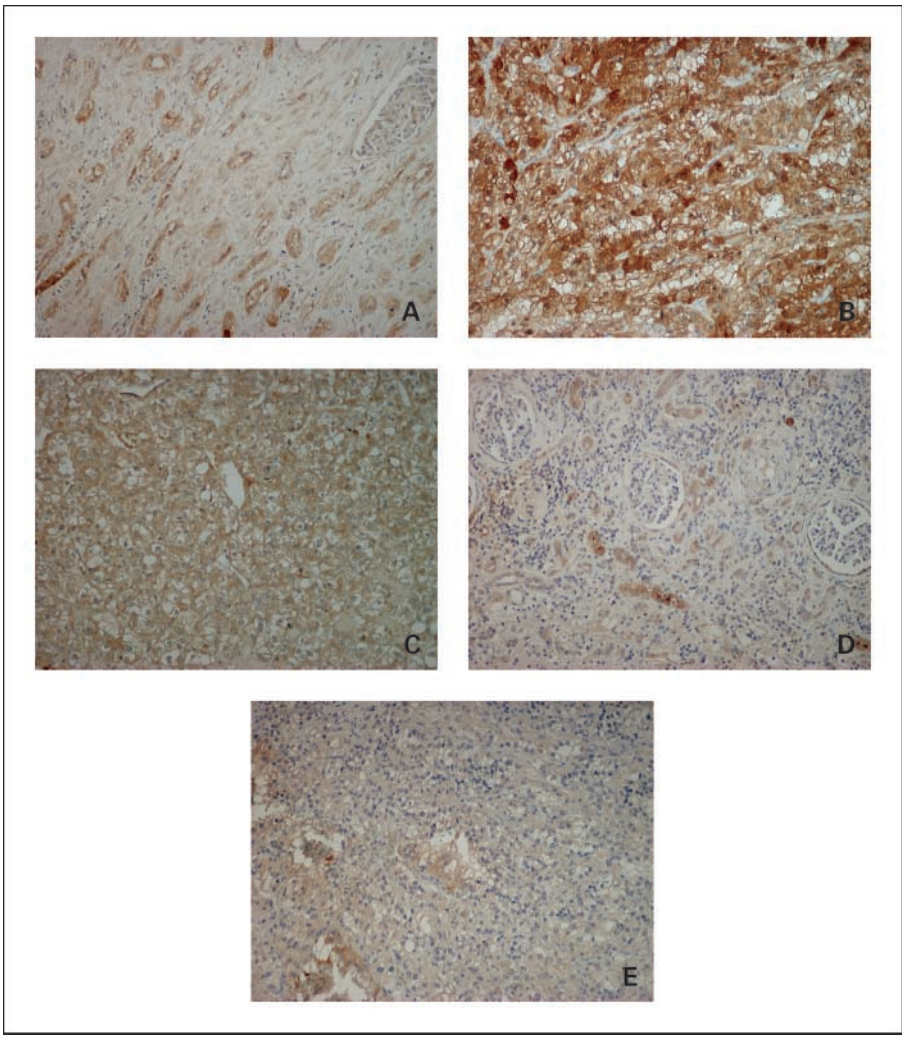

Fig. 2. Immunohistochemical staining of HGFR/c-Met and pY1349 HGFR/c-Met in RCC and adjacent normal renal tissue. *A,* HGFR/c-Met expression in normal renal tissue. *B,* HGFR/c-Met expression in RCC tissue. *C,* expression of pY1349 HGFR/c-Met in tumor tissue. *D,* expression of pY1349 HGFR/c-Met in normal renal tissue. *E,* expression of pY1234/pY1235 HGFR/c-Met in cancer cells.

Cell culture. Human renal carcinoma ACHN cells (12) were cultured in MEM supplemented with nonessential amino acids and 10% fetal bovine serum (Life Technologies, Rockville, MD). For examination of the tyrosine phosphorylation of HGFR/c-Met, cells grown in 6-cm dishes were serum starved with MEM supplemented with nonessential amino acids and 0.1% bovine serum albumin overnight. The medium was then replaced with fresh MEM containing nonessential amino acids and 0.1% bovine serum albumin, and cells were either left unstimulated or stimulated with 100 ng/mL recombinant human HGF (R&D Systems, Minneapolis, MN) for indicated periods.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were done as described previously (22). In brief, cells were lysed in NP40 lysis buffer and HGFR/c-Met was immunoprecipitated with anti-human HGFR/c-Met antibody and separated by SDS-PAGE. Proteins were electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and the blots were probed with indicated antibodies. Between two different probing, stripping was done as described previously (23).

Immunohistochemistry. Immunohistochemistry was done on formalin-fixed and paraffin-embedded archival tissues. Sections (5-μm thick) were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was done at 95°C for 40 minutes in 0.01 mol/L sodium citrate buffer (pH 6.0). All sections were then immersed in 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Sections were incubated with either anti-HGFR/c-Met antibody (dilution, 1:350), anti-pY1234/pY1235 HGFR/c-Met antibody (1:10), anti-pY1349 HGFR/c-Met antibody (1:10), or anti-Ki-67 antibody (1:100) overnight at 4°C. The sections were washed extensively and then treated with peroxidase using the labeled polymer method of DAKO EnVision+ Peroxidase, Mouse (DAKO Corp., Carpinteria, CA) for 60 minutes. The immunoreactive proteins were visualized by using a liquid 3,3′-diaminobenzidine substrate kit (Zymed Laboratories), and sections were counterstained in hematoxylin. A consecutive section from each sample processed without the primary antibody was used as a negative control.

Evaluation and statistical analyses. Evaluation of HGFR/c-Met expression was assessed semiquantitatively according to a previous report (16). Briefly, specimens were considered to be positive when >50% of cancer cells showed higher staining than normal kidney. To examine the phosphorylated HGFR/c-Met expression, the percentages of positively stained cells were measured separately in cancer cells and normal adjunct tubular cells. If the percentage of stained cancer cells was higher than that of normal tubular cells, the presence of both pY1234/pY1235 HGFR/c-Met and pY1349 HGFR/c-Met was considered as positive expression. Proliferating cells, of which nuclei were positively stained with anti-Ki-67 antibody, were counted, and the proliferation index was determined as the ratio of proliferating cells to all tumor cells as a percentage (at least 500 tumor cells were included). These cells were examined using a Nikon E400 microscope, and digital images were captured (Nikon DU100, Tokyo, Japan) at ×200 magnification. In addition, we used a computer-aided image analysis system (Win ROOF version 5.0, Mitani Corp., Fukui, Japan) to calculate the statistical variables. Slides were blindly evaluated twice at different times by two investigators (Y.M. and S.K.) who were blinded to the

pathologic characteristics, and average levels were used for statistical analyses. All data were expressed as median and range because the clinical data were not normally distributed. The Mann-Whitney *U* test was done for continuous variables. The Fisher's exact test was used for categorical comparison of the data. Spearman correlation coefficients were used to evaluate relationships between continuous variables. All statistical tests were two sided, and significance was defined as $P < 0.05$. All statistical analyses were done on a personal computer with the statistical package StatView for Windows (version 5.0; Abacus Concepts, Inc., Berkeley, CA).

Results

Clinicopathologic features. The clinicopathologic features observed in this study are summarized in Table 1. The mean (SD, median) age at operation was 61.0 years (12.2, 61), and 86 males and 26 females were included. Among 114 patients, 86 (75.4%) had low pT stage (pT1 and pT2) and 28 (24.6%) had high pT stage (pT3 and pT4). Nine patients had lymph node metastasis, and 17 patients had distant metastasis. So, when survival analyses were done, 20 patients were classified into the metastasis group because 6 patients had both lymph node and distant metastasis. With regard to nuclear grade, there were 41 tumors of grade 1, 57 of grade 2, 15 of grade 3, and only 1 of grade 4. The mean (SD, range) follow-up period was 41.4 months (30.1, 1-164), and 21 (18.4%) patients died of RCC during this time.

Kinetic study of tyrosine phosphorylation of HGFR/c-Met in ACHN cells. Phosphorylation site-specific antibodies are useful to predict the activation status of protein kinases *in vivo*. Phosphorylation of tyrosine residues at 1234 and 1235 (Y1234/Y1235) is critical for HGFR/c-Met tyrosine kinase activation, whereas phosphorylation of Y1349 creates a docking site for SH2 domain-containing downstream signaling molecules (5). These tyrosine residues are autophosphorylated on activation of HGFR/c-Met. We examined the kinetics of tyrosine phosphorylation at these residues in a human renal carcinoma cell line, ACHN cells. As shown in Fig. 1, phosphorylation of Y1349 was observed at 5 minutes after HGF treatment and continued for 120 minutes. In contrast, maximal phosphorylation of Y1234/Y1235 was observed at 30 minutes after which point it rapidly diminished. Total tyrosine phosphorylation lasted longer than pY1234/pY1235 and pY1349, suggesting that other phosphorylated tyrosine residues may be more stable than Y1234/Y1235 or Y1349. The total amount of HGFR/c-Met protein was decreased at 180 minutes after HGF treatment. This was probably due to the degradation by proteasome following internalization (24) because the proteasome inhibitor MG-126 protected this decrease in HGFR/c-Met (data not shown).

Expression of HGFR/c-Met and phosphorylated HGFR/c-Met in RCC tissues. Representative examples of HGFR/c-Met staining in normal kidney tissues and cancer cells are shown in Fig. 2A and B, respectively. HGFR/c-Met was localized to the cytoplasm and membranes of tubular cells of normal kidney and cancer cells. Median (range) rate of positively stained cancer cells was 64.3% (12.3-87.4%), and 73 tumors were judged as positive for HGFR/c-Met expression. On the other hand, pY1349 HGFR/c-Met was mainly detected in cancer cell membranes (Fig. 2C) and showed a median (range) positive staining rate of 14.3% (0-64.3%). Furthermore, staining of pY1349 HGFR/c-Met was lower than that of HGFR/c-Met over all cancer cell specimens. In normal kidney, pY1349 HGFR/c-Met was detected in cell membranes of tubular cells (Fig. 2D), with 46 (40.4%) specimens judged as positive for pY1349 HGFR/c-Met. In contrast, the immunopositive rate of pY1234/pY1235 HGFR/c-Met was remarkably low (0%, 0-5.2%) in RCC tissues (Fig. 2E). Due to the low expression rate and short period of pY1234/

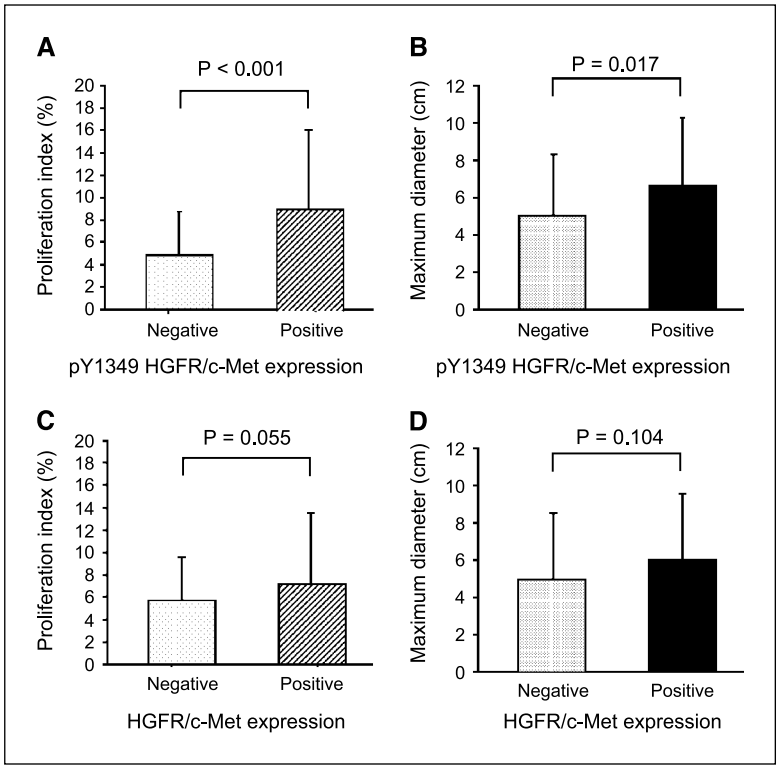

Fig. 3. Proliferation index (*A*) and maximum diameter of the primary tumor (*B*) according to the status of pY1349 HGFR/c-Met. Proliferation index (*C*) and maximum diameter of the primary tumor (*D*) according to the status of HGFR/c-Met.

pY1235 HGFR/c-Met expression in the kinetic study (Fig. 1), we assessed the presence of phosphorylated HGFR/c-Met by the presence of pY1349 HGFR/c-Met throughout the study.

Clinical significance of HGFR/c-Met expression and phosphorylated HGFR/c-Met expression. The relationship between clinicopathologic features and the expression of pY1349 HGFR/c-Met is shown in Table 1. Expression of pY1349 HGFR/c-Met was positively associated with pT stage ($P < 0.001$), lymph node metastasis ($P = 0.014$), distant metastasis ($P = 0.021$), and tumor grade ($P = 0.004$). However, it was not associated with gender or age at diagnosis. Furthermore, as shown in Fig. 3A, the proliferation index in pY1349 HGFR/c-Met-positive tumors (8.9 $\pm$ 7.1%) was significantly higher ($P < 0.001$) than that in negative tumors (4.8 $\pm$ 3.9%). Accordingly, the maximal diameter of pY1349 HGFR/c-Met-positive tumors was significantly larger ($P = 0.017$) than that of immunonegative tumors (6.6 $\pm$ 3.7 cm versus 5.0 $\pm$ 3.3 cm; Fig. 3B). However, HGFR/c-Met expression itself was not significantly correlated to the proliferation index (positive, 7.2 $\pm$ 6.3% versus negative, 5.1 $\pm$ 4.5%; $P = 0.055$; Fig. 3C) nor to the maximal diameter of tumors (6.1 $\pm$ 3.5 cm versus 4.9 $\pm$ 3.6 cm; $P = 0.104$; Fig. 3D).

Survival analyses. Next, Kaplan-Meier curves of cause-specific survival according to the positive staining rates of HGFR/c-Met and pY1349 HGFR/c-Met were examined. Although no association was found between HGFR/c-Met expression and cause-specific survival ($P = 0.142$; Fig. 4A), positive expression of pY1349 HGFR/c-Met was recognized as a significant and useful predictive factor by log rank test ($P < 0.001$; Fig. 4B). In addition, high pT stage, presence of metastasis, and high grade of tumor were also significant predictors by univariate analysis (Table 2). When multivariate Cox analysis was done, nuclear grade had no independent prognostic effect in our study population ($P = 0.325$). Although a trend was noted between high pT stage and cause-specific survival in our multivariate analysis model, pT stage did not reach statistical significance ($P = 0.085$). On the other hand, presence of metastasis and pY1349 HGFR/c-Met expression were independent predictors of cause-specific survival (odds ratio, 6.43; 95% confidence interval, 2.25-18.39; $P < 0.001$ and odds ratio, 2.94; 95% confidence interval, 1.12-7.72; $P = 0.028$, respectively; Table 2).

Discussion

In the present study, positive expression of pY1349 HGFR/c-Met was positively associated with tumor grade, stage, and size as well as cancer cell proliferation in patients with conventional RCC. In addition, pY1349 HGFR/c-Met expression was a significant and independent predictor for cause-specific survival by multivariate analysis. In contrast, pY1234/pY1235 HGFR/c-Met expression was not detected in the majority of specimens. Although the expression of phosphorylated HGFR/c-Met in human RCC tissues has not been reported in the literature, several investigators have implicated HGFR/c-Met expression in the carcinogenesis and progression of RCC (15, 25, 26). However, contrary to our hypothesis, our results showed no association between HGFR/c-Met expression and clinicopathologic findings or survival, supporting a study by Nakopoulou et al. (27). This discrepancy between our study and others is probably due to the differences in antibodies used for experiments or to patient characteristics, including number of patients and histologic variation. In recent years, several investigators have stressed that activation of HGFR/c-Met is important for tumor growth and progression in other cancers (19, 20). For example, Inoue et al. (19) reported that phosphorylated Met staining was detected only in gastric cancer tissues and not in normal tissue and that this expression was associated with increased cell proliferation. In addition, Ma et al. (20) observed the preferential expression of phosphorylated Met at the tumor invasive front in non–small cell lung cancer tissues and reported an association with the biology and biochemistry of the tumor. Our findings from the present study support their results and opinions, and we also speculate that phosphorylation of HGFR/c-Met is crucial for tumor development in RCC.

The mechanism underlying the distinct kinetics of tyrosine phosphorylation between Y1234/Y1235 and Y1349 is not clear. After ligand binding, receptor tyrosine kinases are dimerized, autophosphorylated, and then involved with downstream signaling molecules followed by rapid internalization and degradation. Y1349 is a multidocking site for SH2-containing proteins. It is therefore possible that phosphorylated Y1349 may act to protect the protein from degradation by bound signaling proteins. Alternatively, protein tyrosine phosphatases, which associate with activated HGFR (28), may preferentially dephosphorylate Y1234/Y1235 rather than Y1349. However, treatment of ACHN cells with the phosphatase inhibitor orthovanadate failed to increase phosphorylation of Y1234/Y1235 and Y1349 (data not shown), suggesting that tyrosine phosphatases were not responsible for the different kinetics of tyrosine phosphorylation. Interestingly, detection of phosphotyrosine in the kinase domain was less frequent than

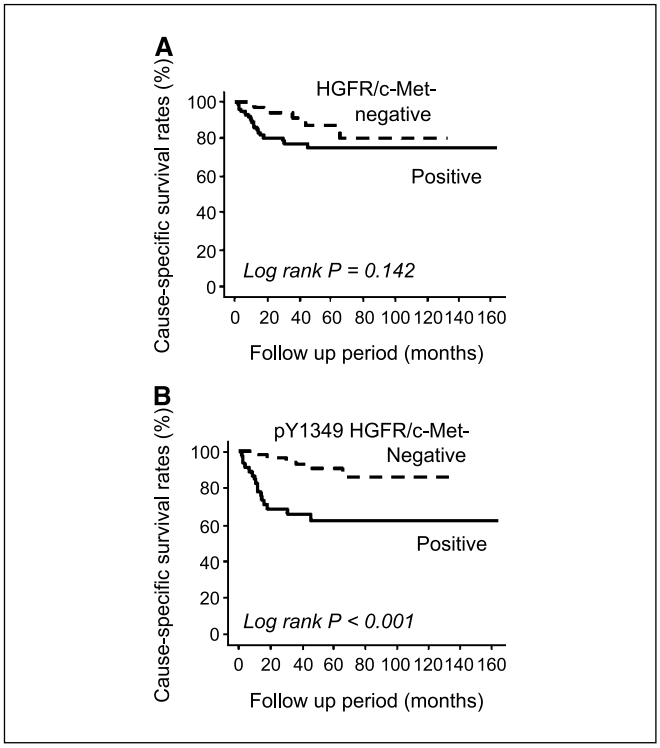

Fig. 4. Kaplan-Meier recurrence to cause-specific survival curves according to the HGFR/c-Met expression (*A*) and pY1349 HGFR/c-Met expression (*B*).

Table 2. Cox regression analyses for cause-specific survival

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
pT stage				
High (pT3 + pT4)	10.42 (4.21-25.80)	<0.001	2.72 (0.87-8.52)	0.085
Metastasis				
Presence	14.82 (6.06-36.27)	<0.001	6.43 (2.25-18.39)	<0.001
Grade				
High (grade 3 + 4)	7.00 (2.81-17.40)	<0.001	1.69 (0.59-4.84)	0.325
HGFR/c-Met				
Positive	0.48 (0.18-1.31)	0.151	—	—
pY1349 HGFR/c-Met				
Positive	4.91 (4.91-12.58)	<0.001	2.94 (1.12-7.72)	0.028

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.

phosphotyrosine located outside of the kinase in lung cancer (20), and our data reproduced this observation. Thus, it seems likely that detection of autophosphorylated HGFR/c-Met directed to phosphotyrosines located outside of the kinase domain may be useful to cover the larger population of tumor cells having activated HGFR/c-Met.

In conclusion, activation of HGFR/c-Met plays an important role for tumor growth and progression of RCC. In human RCC, pY1349 HGFR/c-Met is a better indicator of phosphorylated HGFR/c-Met than pY1234/pY1235 HGFR/c-Met. In addition, detection of pY1349 HGFR/c-Met expression is an excellent predictor for the prognosis of patients with sporadic conventional RCC patients.

Acknowledgments

We thank Takumi Shimogama and Yoshikazu Tsuji for their excellent assistance.

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