Expression of *c-met*/HGF Receptor in Human Non-small Cell Lung Carcinomas *in vitro* and *in vivo* and Its Prognostic Significance

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The expression of *c-met*/HGF receptor was evaluated in non-small cell lung cancers (NSCLC) by western blot analysis of 11 established cell lines and 104 surgically resected tissues. All cancer cell lines (eight adenocarcinomas, two squamous cell carcinomas and a large cell carcinoma) showed strong *c-met* protein bands of 145 kDa and 170 kDa. Moreover, *c-met* protein was demonstrated in 34 (72.3%) of 47 surgically resected adenocarcinomas, 20 (38.5%) of 52 squamous cell carcinomas and 3 of 5 others, and the results were mostly confirmed immunohistochemically in formalin-fixed and paraffin-embedded tumors of the same case. Although squamous cell carcinomas showed relatively high *c-met* protein expression in established cell lines, more adenocarcinomas than squamous cell carcinomas showed *c-met* protein expression in the original cancers. Furthermore, two cell lines used in this study originated from primary cancers negative for *c-met* protein expression, suggesting that *c-met* protein expression might be influenced by cultivation. Furthermore, clinicopathological study revealed that NSCLC with *c-met* protein expression tended to be in a higher pathological tumor stage and to have a worse outcome than those without such expression. In conclusion, *c-met* protein is expressed in cell lines and primary tumors of NSCLC, and this phenomenon is probably closely related to the aggressive behavior or progression of NSCLC, especially of adenocarcinomas.

Key words: *c-met* — HGF — Western blotting — Non-small cell lung carcinoma — Progression

The *c-met* proto-oncogene encodes a transmembrane tyrosine kinase of the growth factor receptor, of which the ligand is hepatocyte growth factor (HGF).¹⁾ HGF is a potent growth factor found in the serum of partially hepatectomized rats and is a heterodimeric polypeptide composed of a 69-kDa α -chain and a 34-kDa β -chain, linked by one disulfide bridge.^{2,3)} In the process of regeneration of many organs, HGF is produced by various mesenchymal cells such as fibroblasts, endothelial cells and macrophages, and has pleiotropic effects as a mitogen, motogen and morphogen on epithelial cells of target organs.⁴⁾ In addition to hepatocytes, renal tubular epithelial cells, keratinocytes, melanocytes, alveolar epithelial cells and bronchial epithelial cells are target cells for HGF.³⁾

On the other hand, *c-met*/HGF receptor is a heterodimeric molecule composed of a 50-kDa α -subunit and a 145-kDa β -subunit and is expressed not only in hepatocytes, but also in a variety of other cells and tissues.⁵⁾ In the epithelial cell surface, there are high-affinity HGF receptors at the rate of 40–720 sites/cell, which play an important role in intracellular signal transduction of HGF.⁶⁾ In the regenerative process after liver injury, *c-met*/HGF receptors are rapidly down-regulated soon after the increase of HGF mRNA, then DNA synthesis

of liver cells starts.⁷⁾ Therefore, the down-regulation of *c-met*/HGF receptor is a critical phenomenon in the target cells for HGF.

In the development of the lung, the levels of HGF and its receptor are very low during late gestation and in neonates and then increase during maturation of the lung.⁸⁾ As HGF is produced by fibroblasts and endothelial cells of the alveolar wall as well as by macrophages in the alveolus and *c-met*/HGF receptors are expressed on the plasma membrane of the alveolar type II cells and the bronchial epithelium, they may play important role in the development and maturation of the lung and the regenerative process after injury as a paracrine mechanism.^{4, 8–14)} Recently, it has become evident that HGF and its receptor are involved as either paracrine or autocrine in carcinogenesis of the lung.^{12, 15)} We report here the expression of *c-met* protein in non-small cell lung cancers (NSCLC), in both cell lines and fresh tumor tissues, and the clinicopathological significance of *c-met* protein expression in NSCLC.

MATERIALS AND METHODS

Lung cancer cell lines A total of 11 NSCLC cell lines consisting of eight adenocarcinomas (JCRB0081 (RERF-LC-MS),¹⁶⁾ JCRB0815 (ABC-1),¹⁷⁾ JCRB0811 (RERF-LC-OK), JCRB0814 (VMRC-LCD), GLAD-1, 2, 3, 4), two squamous cell carcinomas (GLSQ-1, 2) and a large

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Table I. Summary of Non-small Cell Lung Cancer Cell Lines Used

Name	Clinicopathological data of original cancers					Biological data of cell lines	
	Histology	Sex	Age	c-met protein		Doubling time (h)	DNA ploidy
				WB ^{a)}	IHS ^{b)}		
JCRB0815	Ad.	— ^{c)}	—	—	—	33	4.4
GLLA-1	Large	F	48	ND ^{d)}	(+)	43	4.4
GLSQ-1	Sq., well	M	58	(—)	(—)	ND	ND
JCRB0811	Ad.	—	—	—	—	22	5.4
GLAD-2	Ad., poor.	M	38	(+++)	(+)	34	ND
GLAD-4	Ad., poor.	F	62	ND	ND	ND	3.7
GLSQ-2	Sq., poor.	M	63	(++)	(—)	22	4.6
JCRB0814	Ad.	—	—	—	—	ND	ND
JCRB0081	Ad.	—	—	—	—	40	4.2
GLAD-3	Ad., poor.	M	49	ND	ND	24	6.9
GLAD-1	Ad., mod.	M	67	(—)	(—)	ND	ND

a) WB, Western blot analysis.

b) IHS, Immunohistochemistry.

c) —, Not available.

d) ND, Not done.

cell carcinoma (GLLA-1) were used in this study (Table I). Cell lines JCRB0081, JCRB0815, JCRB0811 and JCRB0814 were provided by the Japanese Cancer Research Resources Bank (Tokyo) and were cultivated in Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS), non-essential amino acids (0.1 mmol of L-alanine, 0.1 mmol of L-asparagine, 0.1 mmol of L-aspartic acid, 0.1 mmol of L-glutamic acid, 0.1 mmol of L-proline, 0.1 mmol of L-serine and 0.1 mmol of L-glycine) and 4 mM HEPES. GLSQ, GLAD and GLLA cell lines were established from surgically resected tumor tissues in our laboratory. These cell lines were primarily cultivated in RPMI 1640 medium supplemented with 10% FCS, 25 mmol of L-glutamic acid and penicillin G (100 IU/ml). Clinical and biological information about these cell lines is summarized in Table I.

NSCLC tissues NSCLC tissues, which were freshly obtained from 104 patients at surgery in Gunma University Hospital and its affiliated hospitals from 1990 to 1994, were frozen and kept at -80°C until use. The ages of the patients ranged from 34 to 85 years (mean 66.2 years). The male-to-female ratio was 10 : 1 for squamous cell carcinomas and 2 : 1 for adenocarcinomas. All lung cancers were also fixed in 15% formalin and embedded in paraffin for routine pathological examination and for immunohistochemical study.

Western blot analysis Each NSCLC cell line was harvested at the exponential growth phase and the cell pellet was immediately homogenized in 1 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 150 mmol of NaCl, 0.5% Triton X-100 and 0.2 mmol of phenylmethylsulfonyl fluoride with a glass homogenizer. The homogenate in a

1.5-ml Eppendorf tube was centrifuged at 10,000 rpm for 15 min at 4°C and the clear supernatant was used for western blot analysis after determination of the protein concentration with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). The soluble protein was also extracted from frozen NSCLC tissues and normal bronchial mucosal tissues freshly removed from the bronchus in the same manner and used for western blot analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 7.5% gel under reducing conditions according to the method of Laemmli.¹⁸⁾ A total of 100 μg of extracted protein per lane was loaded on SDS-PAGE mini-gel, and the separated protein bands were electrophoretically transferred onto a polyvinylidene difluoride membrane by Trans-Blot SD Semi-Dry Electrophoretic Transfer (Bio-Rad Laboratories, Richmond, CA). As a positive control, 25 μg of extracted protein from GLSQ-1 per lane was added to each SDS-PAGE gel. Then the membrane was immersed in 20 mM Tris-HCl-buffered saline (TBS) containing 5% skim milk at 4°C for 12 h to prevent nonspecific staining. The membrane was washed with TBS containing 0.025% Tween 20 (TBS-T), then incubated with polyclonal rabbit anti-human c-met antibody (sc-10, Santa Cruz Biotechnology, Inc., Delaware, CA) at a 1 : 100 dilution in TBS containing 1% bovine serum albumin for 2 h at room temperature. After having been washed with TBS-T three times, the membrane was incubated with anti-rabbit goat IgG conjugated with horseradish peroxidase (Dako Japan, Kyoto) at a 1 : 1,500 dilution for 1 h at room temperature. The membrane was washed extensively with TBS-T, then an enhanced chemiluminescence

kit (Amersham International, Buckinghamshire, England) was used for development according to the manufacturer's instructions.

For the evaluation of c-met protein, a 145-Da c-met protein band was densitometrically compared with the positive control band. If the intensity was stronger than that of the positive control, the band was judged as strongly positive (+++). The distinction between moderate (++) and slight (+) positivity was at the 50% level of band intensity compared with the positive control. If the band was not detected, it was judged as negative (-).

In order to study the phenomenon of autocrine growth in NSCLC, western blot analysis for HGF was also carried out the 11 NSCLC cell lines. Antibodies to α and β subunits of HGF were obtained from IBL Laboratories (Gunma) and used at a 1 : 50 dilution. The following western blot analysis was the same as that used for c-met protein detection.

Immunohistochemical examination for c-met protein For immunohistochemical confirmation of the results of western blot analysis, 3- μ m thick paraffin sections were cut from the formalin-fixed and paraffin-embedded tumor tissues of the 104 NSCLC. Immunoperoxidase staining by the avidin-biotin-peroxidase complex method was performed with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The sections were deparaffinized with xylene, washed in phosphate-buffered saline (pH 7.4), and incubated for 1 h with normal goat serum to block nonspecific binding of the antibody. The sections were exposed to polyclonal rabbit anti-human c-met antibody (sc-10, Santa Cruz Biotechnology) and anti-c-met antibody (#18321, IBL Laboratories) at a 1 : 100 dilution overnight at 4°C. Then they were incubated with biotinylated second antibody for 1 h at room temperature, and with the Vectastain ABC reagent for 1 h. For visualization, they were immersed in 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% H₂O₂ for 5 min, and counterstained lightly with hematoxylin.

RESULTS

Western blot analysis clearly demonstrated two main bands of c-met protein at molecular sizes of 145 kDa and 170 kDa in all NSCLC cell lines investigated (Fig. 1). The intensity of the 145 kDa-band was always stronger than that of the 170-kDa band. Moreover, the main band of three cell lines, GLLA-1, JCRB0081 and JCRB0811, was slightly larger in size than 145 kD. In Table I, all cell lines used in this study are arranged in the order of the intensity of the c-met protein band; JCRB0815 showed the strongest c-met protein band. Moreover, two squamous cell carcinoma lines also showed relatively high c-met protein expression. On the other hand, western blot

Fig. 1. Western blot analysis of c-met protein in various non-small cell lung cancer cell lines. The majority of cell lines showed a strong 145-kD band and its precursor band. In lanes, 3, 4 and 6, the main band was slightly larger than 145 kD in size. Lane 1: GLSQ-1, lane 2: GLSQ-2, lane 3: GLLA-1, lane 4: JCRB0081, lane 5: JCRB0815, lane 6: JCRB0811, lane 7: JCRB0814.

Fig. 2. Western blot analysis of c-met protein in various primary non-small cell lung cancers. Primary adenocarcinomas were examined for c-met protein expression by western blot analysis and the grade of expression was divided into strong (lane 3), moderate (lanes 1, 4, 5), slight (lanes 2, 7) and negative (lane 6). Lane 8 was the positive control (GLSQ-1).

analysis failed to detect a specific band for HGF in any of the lung cancer cell lines (data not shown).

By western blot analysis of surgically resected NSCLC, a 145 kDa c-met protein band was detected in 56 (54%) of the 104 NSCLC tissues. However, the 170-kDa band was usually weak and sometimes not detected. In the western blot analysis of surgically resected NSCLC, there were more nonspecific bands, not due to c-met protein, than in the analysis of NSCLC cell lines (Fig. 2). In addition, no specific c-met protein band was observed by western blot analysis using normal bronchial mucosal tissue. The relationship between c-met protein expression and histological subtype of NSCLC is summa-

Table II. Western Blot Analysis of c-met Protein in Non-small Cell Lung Carcinomas

Cancer type	Band intensity			
	+++	++	+	-
Adenocarcinoma (34/47) ^{a)} (72.3%)	9 (19.1%)	6 (12.8%)	19 (40.4%)	13 (27.7%)
Squamous cell carcinoma (20/52) (38.5%)	0 (0%)	8 (15.4%)	12 (23.1%)	32 (61.5%)
Others (2/5) (40%)	0 (0%)	2 (40%)	0 (0%)	3 (60%)

a) (The number of c-met protein-positive cancers)/(the number of cancers investigated).

Table III. Relationship between c-met Expression and the Stage of Lung Carcinomas

c-met protein expression	Stage			
	I	II	III	IV
+++	1 (1) ^{a)}	1 (1)	6 (6)	1 (1)
++	6 (4)	1 (0)	5 (1)	3 (0)
+	19 (13)	3 (1)	7 (4)	1 (1)
-	22 (8)	8 (2)	12 (0)	2 (2)

a) Numbers in parenthesis indicate the number of adenocarcinomas.

Fig. 3. Actual survival of patients with adenocarcinoma according to the grade of c-met protein expression (Kaplan-Meier method). Based on western blot analysis for c-met protein, the patients with adenocarcinoma were divided into three groups; negative (—; n=12), slightly to moderately positive (---; n=24) and strongly positive (.....; n=7).

Fig. 4. Immunohistochemical demonstration of c-met protein in a primary adenocarcinoma of the lung. Infiltrating adenocarcinoma cells were diffusely stained for c-met protein only in the cytoplasm. Lightly counterstained with hematoxylin.

In this series of western blot analyses, the original cancers of four cell lines, GLSQ-1, 2 and GLAD-1, 2, were examined for c-met protein expression and no c-met protein expression was found in two (GLSQ-1 and GLAD-1) (Table I).

Table III shows the relationship between c-met protein expression and pathological stage of the lung cancers investigated in this study. There was a tendency that the higher the stage of NSCLC, the stronger was the intensity of the c-met protein band determined by western blot analysis. In adenocarcinomas, there was a strong similar tendency. Furthermore, the actual survival of patients with adenocarcinoma was calculated 4 years after the surgery by the Kaplan-Meier method and was compared with the degree of c-met protein expression (Fig. 3).

ized in Table II. Adenocarcinomas expressed c-met protein more frequently than squamous cell carcinomas. In fact, strong c-met protein expression densitometrically evaluated as strong (++++) was seen only in adenocarcinomas. In contrast, more than half of the squamous cell carcinomas failed to show any c-met protein expression.

Adenocarcinoma patients with high c-met protein expression showed a worse outcome than those without c-met expression ($P < 0.01$).

Immunohistochemical study revealed c-met protein in 56 (54%) of the 104 NSCLC, which consisted of 77% (36/47) of the adenocarcinoma cases and 37% (19/52) of the squamous cell carcinoma cases. In a comparison of the immunohistochemical results with those of western blot analysis, the results were identical in most cases, but 17 tumors (16.3%) showed a discrepancy between the results obtained by the two methods. The discrepancy rate was higher in squamous cell carcinoma (21%) than in adenocarcinoma (13%). Immunoreactivity for c-met protein was diffusely present in the cytoplasm of cancer cells and showed marked diversity from area to area and case to case (Fig. 4). In general, adenocarcinomas tended to have stronger immunoreactivity than squamous cell carcinomas. No immunoreactivity for c-met protein was observed in any normal epithelia of alveolus and bronchus.

DISCUSSION

HGF and its receptor are well known to have an important stimulatory effect on normal and neoplastic human bronchial and alveolar epithelial cells.^{4, 8-14} Previous studies have revealed the expression of the *c-met* gene in human lung cancers and their cell lines, especially of NSCLC, but rarely in small cell carcinomas.¹⁹⁻²¹ In this study, western blot analysis revealed c-met protein expression not only in all NSCLC cell lines and many primary NSCLC, but also more frequently and more strongly in adenocarcinomas than in squamous cell carcinomas of primary NSCLC. In *in vitro* and *in vivo* studies according to the histologic subtype of NSCLC, Liu and Tsao found a higher percentage of *c-met* gene expression in adenocarcinomas than squamous cell carcinomas.^{20, 21} We broadly confirmed their results by western blot analysis using a large number of freshly obtained NSCLC tissues and established cell lines. Moreover, we found a c-met band larger in size than the normal 145 kD in three NSCLC cell lines. This phenomenon is likely to be the result of glycosylation of c-met protein, as pointed out by Di Renzo *et al.*,²² but further studies are necessary to reach a conclusion.

HGF expression in human alveolar and lung cancer cells is a controversial issue.¹²⁻¹⁵ HGF has been demonstrated in NSCLC cell lines and suggested as either an autocrine or a paracrine factor for lung cancer development.²³ In a small cell carcinoma cell line, SBC-5, simultaneous expression of a unique two-kringle smaller variant of HGF and *c-met*/HGF receptor has been suggested to act as an autocrine mechanism in either development or progression of lung cancer cells.²⁴ However, most

reports have suggested HGF and c-met interaction as a paracrine mechanism in lung cancer cells, as well as normal bronchial and alveolar cells.¹³⁻¹⁵ As our western blot analysis failed to detect HGF protein in NSCLC cell lines, further studies are necessary to permit a conclusion.

In NSCLC, various growth factors and their receptors play important roles in the development and maintenance of the cancer cells.^{20, 21, 25} Liu and Tsao have extensively studied the expression of proto-oncogenes and growth factor/receptors in NSCLC and found that the expression of the *c-erbB-2* and *c-met* genes is closely related to the histological subtypes of NSCLC, especially of adenocarcinomas.²¹ Although epidermal growth factor receptor and transforming growth factor- α (TGF- α) are thought to function as a potential autocrine loop in NSCLC, their expression is a common event in NSCLC and they may participate in tumor growth without necessarily influencing tumor progression or histology.^{21, 25}

Several previous studies have shown that the overexpression of the *c-met* gene is closely related to growth advantage and the pathological stage of the tumor in many cancers such as hepatocellular, ovarian, thyroid, prostatic, pancreatic and breast carcinomas.^{22, 26-32} However, no report has appeared concerning this issue in lung carcinomas. In this study, we clearly showed that c-met protein expression might be closely associated with pathologic stage and the patient's prognosis in NSCLC. These results provide evidence that paracrine or autocrine growth mechanisms regulated by HGF and c-met interaction have an important role in the proliferation and progression of NSCLC. However, a recent immunohistochemical study has revealed that c-met protein expression is upregulated during the early stages of neoplastic promotion, and that a lack or subsequent loss of c-met protein in adenocarcinomas appears to result in a more aggressive behavior in human pancreatic carcinogenesis.³³ In this study, there were several NSCLCs lacking c-met protein expression even in an advanced tumor stage. We speculate that there may be another mechanism of progression of NSCLC, irrespective of upregulation of the *c-met* gene, as suggested for pancreatic adenocarcinoma.

Although c-met protein expression was not very strong in primary squamous cell carcinoma in this study, strong expression of the c-met protein was observed in squamous cell carcinoma cell lines. Moreover, our results indicated a discrepancy in c-met protein expression between a primary tumor and established cell lines. This discrepancy in c-met protein expression between NSCLC *in vivo* and *in vitro* can be attributed to the possible selective growth of cancer cells with c-met protein expression during cultivation because of a growth advantage or more aggressive behavior. Liu and Tsao reported that an NSCLC cell line can be easily established from

original tumor cells with TGF- α and *c-met* gene expression and that tumor cells grown continuously as a cell line often represent a selective subpopulation of the heterogeneous neoplastic cells in the primary tumor,²⁰⁾ suggesting a difference in the tumor cell subpopulation between primary and subsequently cultured or xenotransplanted tumors. In the xenotransplantation of tumors as well as tumor cell cultivation, it is easy to establish cell lines if the tumor cells have an oncogene abnormality that is advantageous for cell growth or tumor progression.³⁴⁾ These facts indicate that cancer cells with *c-met* gene expression may have a growth advantage and have the ability to grow selectively in a new environment, a phenomenon which would be reflected in the patient's outcome.

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In conclusion, the *c-met* protein expression histologically is greater in adenocarcinomas than in squamous cell carcinomas in NSCLC and is closely related to tumor stage and the patient's outcome, indicating that the interaction of HGF and *c-met* might influence the progression of NSCLC.

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