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Clinical significance of immunohistochemical expression of insulin-like growth factor-1 receptor and matrix metalloproteinase-7 in resected non-small cell lung cancer

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Abstract. Insulin-like growth factor-1 receptor (IGF-1R) and matrix metalloproteinase-7 (MMP-7) have been reported to be related to tumor invasion and metastasis in various malignancies. The aim of this study was to evaluate the expression levels of IGF-1R and MMP-7 in resected non-small cell lung cancer (NSCLC) and to examine the relationship of such levels to clinical characteristics and survival. Expression was measured immunohistochemically. The percentage of stained cells was multiplied by the staining intensity. The sample was classified as high when the score was equal or higher than the median value or was otherwise considered to be low. High IGF-1R expression was associated with nodal metastasis and recurrence (P=0.034 and 0.006, respectively). High IGF-1R expression was associated with significantly poorer overall survival than low IGF-1R expression (P=0.011). MMP-7 expression did not significantly correlate with any clinicopathological factor. There was a trend toward slightly, but not significantly poorer survival in patients with MMP-7-high tumors than in those with MMP-7-low tumors (P=0.220). There was no significant correlation between IGF-1R expression and MMP-7 expression (P=0.184). Upon multivariate analysis, IGF-1R expression was independently related to the outcomes of patients with NSCLC. Overexpression of IGF-1R may be a useful predictor of lymph node metastasis, recurrence and post-surgical outcomes in patients with NSCLC.

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Introduction

Primary lung cancer is one of the most common types of cancer worldwide, and non-small cell lung cancers (NSCLCs) account for approximately 85% of all primary lung cancers. Surgical resection is the only potentially curative treatment for patients with early disease. However, the 5-year survival rate after surgical resection remains unsatisfactory. Improved survival of patients with NSCLC requires better clinical predictors of outcomes and of response to specific therapeutic interventions.

Insulin-like growth factor-1 receptor (IGF-1R) is a transmembrane heterotetrametric protein encoded by the *IGF-1R* gene located on chromosome 15q25-q26. IGF-1R promotes oncogenic transformation, growth and survival of cancer cells (1-4). The binding of insulin-like growth factor (IGF)-1 and IGF-2 to the extracellular subunit domain of IGF-1R activates the tyrosine kinase activity of IGF-1R and triggers a cascade of reactions involving signal transduction pathways, including components such as Ras, Raf, mitogen-activated protein kinase and phosphoinositol-3-kinase (PI3K)/AKT/BAD (Bcl-xL/Bcl2-associated death promoter) (5). IGFs are synthesized together with six molecular species of specific binding proteins [IGF binding protein (IGFBP)-1 to -6]. IGFBPs modulate IGF-1 and IGF-2 bioavailability in both circulation and the cellular microenvironment. In several malignancies, IGF-1R overexpression promotes tumor growth, progression, invasion and metastasis (6). Increased metastatic activity was reported in mice after intrasplenic injection of lung cancer cell lines transfected with IGF-1R (7).

Matrix metalloproteinases (MMPs) are a family of highly conserved enzymes that are capable of degrading the extracellular matrix (ECM). Over 25 well-characterized members of this proteinase family have been identified. MMPs play key roles not only in normal processes, but also in tissue remodeling associated with inflammatory disease, cancer invasion and metastasis (8,9). Substantial evidence indicates that overexpression of MMPs correlates with a more aggressive tumor-cell phenotype, as well as with poor outcomes in patients with cancer.

MMP-7 is the smallest (28 kDa) member of the MMP family. It has broad substrate specificity against ECM components and is produced by tumor cells. The functions of MMP-7 include destruction of basement membrane components, which is a crucial event in tumor cell invasion and metastasis. Increased expression of MMP-7 in cancer cells is associated with tumor progression and metastasis in various types of cancer (10,11). To date, few studies have examined the expression of MMP-7 in lung cancer (12-14).

Miyamoto *et al* (15) reported that MMP-7 possesses IGFBP-3 protease activity. MMP-7-induced proteolysis of IGFBP-3 plays a crucial role in regulating IGF-I bioavailability and promotes cell survival. These findings suggest that MMP-7 may augment carcinogenesis and the progression of tumors that express IGF-1R. Adachi *et al* (16) found that IGF/IGF-1R upregulated MMP-7 expression in a gastrointestinal cancer cell line, suggesting that a positive feedback loop involving IGF-1R and MMP-7 may have a part in tumor progression.

The aim of this study was to evaluate the expression levels of IGF-1R and MMP-7 in resected NSCLC, and to examine the relations of such levels to clinical characteristics and survival.

Patients and methods

Patients. This study was performed in 78 consecutive patients with pathological (p)-stage I to III NSCLC who underwent complete tumor resection and nodal dissection without any pre-operative therapy at the Respiratory Center, Yokohama City University Medical Center, between January 1, 2000 and November 30, 2003.

The subjects were 54 men and 24 women with a mean age of 64.7 years (range 19-82; median 65) (Table I). The most common histological type of tumor was adenocarcinoma (57.7%; 45 cases), followed by squamous cell carcinoma (33.3%; 26 cases), large-cell carcinoma (6.4%; 5 cases), typical carcinoid (1 case) and pulmonary blastoma (1 case). The stage of the primary tumor was T1 in 35 patients (44.9%), T2 in 29 (37.1%), T3 in 9 (11.5%) and T4 in 5 (6.4%). Thirty-eight (48.7%) patients had no metastasis to regional lymph nodes (N0), whereas 11 (14.1%) had metastatic involvement of the hilar lymph nodes (N1), and 29 (37.1%) had metastases to the mediastinal nodes (N2, N3). Thirty-two tumors (41.0%) were classified as stage I, 15 (19.2%) were stage II and 31 (39.7%) were stage III. At the end of follow-up, 36 patients (46.1%) were alive and 42 (53.8%) had died.

Histological subgroups were determined according to the World Health Organization classification. Pathological tumor-node-metastasis classification and staging were assigned in accordance with the International Staging System. The mean follow-up was 1,466 days (range 106-3,328). Informed consent was obtained from each patient and the Yokohama City Medical Committee approved this study.

Table I. Patient characteristics.

Characteristics	No. of patients (%)
Total	78 (100)
Age (mean ± SD), years	64.7±10.8
Gender	
Male	54 (69.2)
Female	24 (30.7)
Histological type	
Adenocarcinoma	45 (57.7)
Squamous cell carcinoma	26 (33.3)
Large-cell carcinoma	5 (6.4)
Typical carcinoid	1 (1.3)
Pulmonary blastoma	1 (1.3)
Pathological stage	
I	32 (41.0)
II	15 (19.2)
III	31 (39.7)
Smoking status	
Smoker	56 (71.8)
Non-smoker	22 (28.2)
T-factor	
T1	35 (44.9)
T2	29 (37.2)
T3	9 (11.5)
T4	5 (6.4)
N-factor	
N0	38 (48.7)
N1	11 (14.1)
N2	28 (35.9)
N3	1 (1.3)
Recurrence	
(+)	36 (46.2)
(-)	42 (53.8)

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue specimens were cut into 4-μm thick sections and mounted on slides. The sections were deparaffinized and rehydrated.

For IGF-1R, the slides were heated in a microwave for 10 min in a 10-μmol/l citrate buffer solution at pH 6.0 and cooled to room temperature for 20 min. After quenching the endogenous peroxidase activity with 3% H_2O_2 for 5 min, the sections were incubated for 60 min at room temperature with the primary antibody diluted at 1:100 for IGF-1R (a rabbit polyclonal antibody, clone 1161; Signalway Antibody, Pearland, TX, USA). Peroxidase-Labeled Polymer EnVision+ kit (Dako, Glostrup, Denmark) was used for specific staining.

For MMP-7, after the endogenous peroxidase activity was blocked, the sections were incubated for 90 min at room temperature with the primary antibody for MMP-7 (a mouse monoclonal antibody, clone 141-7B2; Daiichi Fine Chemicals, Toyama, Japan), diluted at 20 μg/ml. Endogenous biotin was blocked by Dako's Biotin Blocking system (Dako), according to the manufacturer's specifications. After rinsing, specific staining was visualized with the use of an LSAB+ system-HRP system (Dako).

Color was produced by the application of 3,3'-diaminobenzidine for 10 min. The sections were counterstained with Meyer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

All sections were scored semi-quantitatively and qualitatively, without knowledge of the clinical data. Expression levels were measured by immunohistochemical analysis on the basis

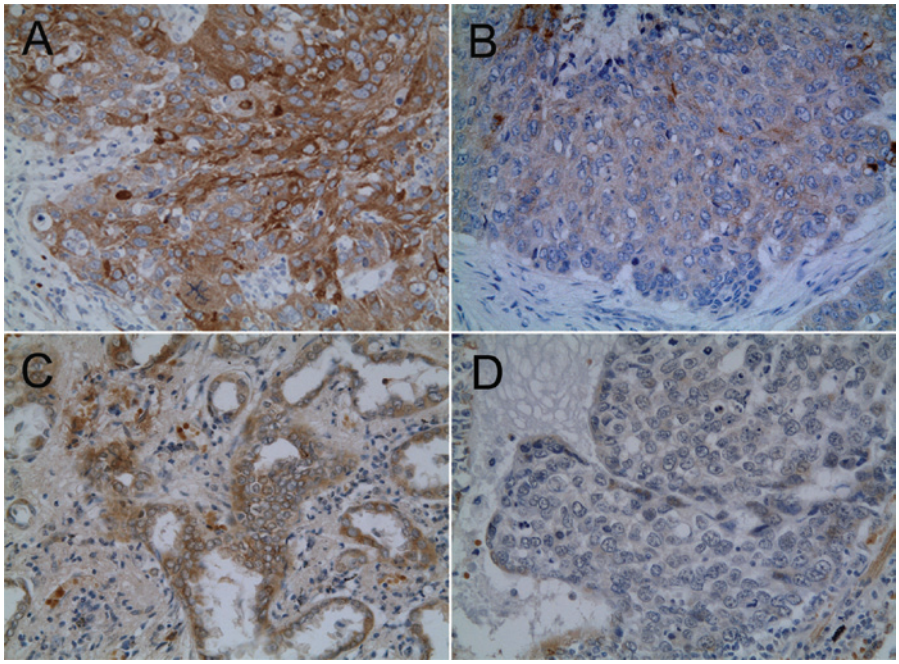

Figure 1. Immunostaining of NSCLC in serial sections. (A) Strong staining (intensity 4) of IGF-1R, and (B) weak staining (intensity 2) of IGF-1R. (C) Strong staining (intensity 4) of MMP-7, and (D) weak staining (intensity 2) of MMP-7.

of staining intensity, scored from 0 to 4 as follows: 0, negative; 1, trace; 2, weak; 3, moderate; and 4, strong. The percentage of stained cells (0-100%) was multiplied by the staining intensity (0-4). The final score ranged from 0 to 400. Staining of the sample was considered high when the score was equal to the median value or higher, or was otherwise considered as low.

Statistical analysis. Univariate analysis was performed by the χ^2 test and Mann-Whitney U test. Continuous data were compared using the Student's t-test. The postoperative survival rate was analyzed by the Kaplan-Meier method, and differences in survival rates were assessed with the log-rank test. A Cox proportional hazard regression model was used for multivariate analyses. Death from any cause was included in the calculation of postoperative survival. Differences were considered significant at $P<0.05$. All statistical manipulations were performed using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Expression of IGF-1R and MMP-7 according to immunohistochemical analysis. Immunohistochemical expression of IGF-1R was detected in the tumor cell membrane. Staining for MMP-7 was characterized by a heterogeneous cytoplasmic pattern (Fig. 1). Among the 78 carcinomas studied, median expression scores were 210 for IGF-1R and 140 for MMP-7. A total of 41 (52.6%) carcinomas were classified as IGF-1R-high, and 42 (53.8%) were classified as MMP-7-high.

Relation between expression of IGF-1R and MMP-7 and clinicopathological factors. High expression of IGF-1R was related to lymph node metastasis (P=0.034) and recurrence (P=0.006). MMP-7 expression status did not significantly correlate with any clinicopathological factor (Table II). There was no significant correlation between the expression of IGF-1R and that of MMP-7 (P=0.184).

Relation between expression of IGF-1R and MMP-7 and overall survival. Overall survival was significantly worse in patients with IGF-1R-high tumors than in those with IGF-1R-low tumors (P=0.011) (Table III, Fig. 2). The 5-year survival rate was 34.1% in patients with IGF-1R-high tumors, as compared to 63.0% in those with IGF-1R-low tumors. Overall survival was slightly, but not significantly, worse in patients with MMP-7-high tumors than in those with MMP-7-low tumors. The 5-year survival rate was 37.5% in patients with MMP-7-high tumors, as compared to 58.3% in those with MMP-7-low tumors (P=0.220) (Fig. 2). Subsequently, we conducted subset analyses to investigate the prognostic significance of IGF-1R and MMP-7. IGF-1R-high was associated with worse overall survival than IGF-1R-low in patients who were male and in those with adenocarcinoma (P=0.022 and 0.016, respectively) (Table IV).

Multivariate analysis of overall survival in patients with NSCLC included the following factors: IGF-1R expression, T-factor, N-factor, gender and MMP-7 expression. Male gender (HR=2.598; 95% CI 1.198-5.638, P=0.016), T2-4 disease (HR=2.540; 95% CI 1.291-4.997, P=0.007) and high expression of IGF-1R (HR=2.322; 95% CI 1.215-4.436, P=0.011) were significantly associated with worse overall survival (Table V).

Discussion

Overexpression of IGF-1R has been reported to promote tumor growth, invasion and metastasis in several types of malignancies (6). Despite previous studies, however, the clinical significance of IGF-1R expression in NSCLC remains unclear. MMP-7 has been reported to have multiple biologic functions related to tumor behavior, such as growth, invasion,

Table II. Characteristics of the NSCLC patients and IGF-1R and MMP-7 expression.

	IGF-1R expression			MMP-7 expression		
	High	Low	P-value	High	Low	P-value
No. of patients	41	37		42	36	
Age (mean ± SD)	64.8±10.0	64.7±11.7	0.960	65.3±10.0	64.3±11.5	0.687
Gender						
Male	27	27	0.496	27	27	0.307
Female	14	10		15	9	
Histological type						
Adenocarcinoma	21	24	0.466	23	22	0.945
Squamous cell carcinoma	17	9		15	11	
Large-cell carcinoma	2	3		3	2	
Other	1	1		1	1	
Smoking status						
Smoker	29	27	0.826	28	28	0.277
Non-smoker	12	10		14	8	
T-factor						
T1	17	18	0.321	19	16	0.986
T2	17	12		15	14	
T3	3	6		5	4	
T4	4	1		3	2	
N-factor						
N0	14	24	0.034	21	17	0.788
N1	6	5		6	5	
N2	20	8		14	14	
N3	1	0		1	0	
Recurrence						
(+)	25	11	0.006	19	17	0.861
(-)	16	26		23	19	

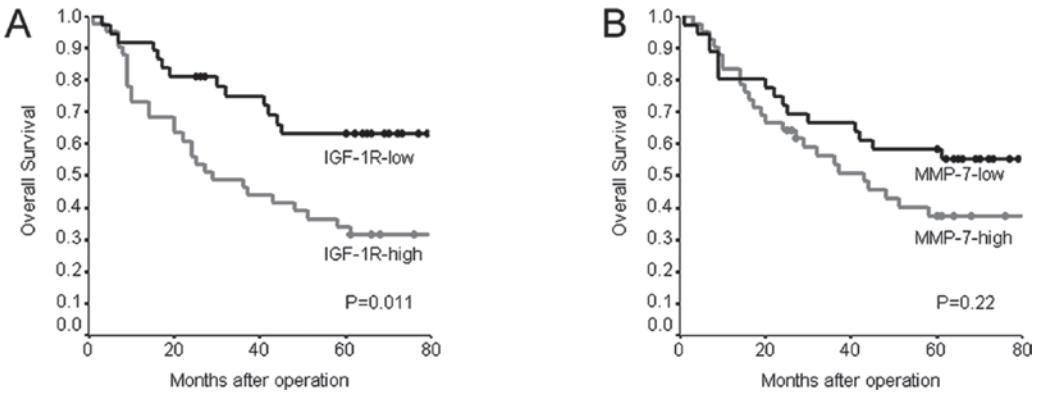

Figure 2. Overall survival of 78 patients with NSCLC. (A) IGF-1R-high vs. IGF-1R-low NSCLC; (B) MMP-7-high vs. MMP-7-low NSCLC.

proliferation and apoptosis. In addition, a relation between MMP-7 expression and postoperative outcomes has been reported (12-14), but definitive evidence is lacking. Therefore, we studied immunohistochemically the expression of IGF-1R and MMP-7 in post-surgical patients with NSCLC.

We assessed the association of IGF-1R and MMP-7 expression with clinicopathological features. Dziadziuszko *et al* (17) found that IGF-1R expression was higher in squamous cell carcinomas than in other histological types and was associated with disease stage. Cappuzzo *et al* (18) reported that a positive IGF-1R expression was significantly associated with squamous cell histology and grade III differentiation. Ludovini *et al* (19) reported that IGF-1R protein overexpression was associated with larger tumor size. Merrick *et al* (20) showed that higher IGF-1R scores were associated with adenocarcinoma and never-smokers. Our results showed that high IGF-1R expres-

Table III. Univariate analysis of overall survival in NSCLC.

Variables	5-year survival rate (%)	P-value
Gender		0.015
Male	40.1	
Female	64.8	
Histological type		0.176
Adenocarcinoma	54.2	
Squamous cell carcinoma	37.5	
T-factor		0.002
T1	67.2	
T2,3,4	32.5	
N-factor		0.280
N0	57.2	
N1,2,3	38.9	
Smoking status		0.109
Smoker	43.6	
Non-smoker	57.7	
IGF-1R expression		0.011
High	34.1	
Low	63.0	
MMP-7 expression		0.220
High	37.5	
Low	58.3	

sion was significantly related to higher N-factor (P=0.034) and recurrence (P=0.006). As for histological type, IGF-1R expression was slightly, but not significantly, higher in squamous cell carcinomas.

Concerning MMP-7 expression, Liu *et al* (12) showed that MMP-7 expression was significantly higher in squamous cell carcinomas than in adenocarcinomas. Leinonen *et al* (13) reported that high MMP-7 expression was related to lower T-factor and well-differentiated tumors; moreover, MMP-7 expression was higher in adenocarcinomas than in other histological subtypes. Sasaki *et al* (14) demonstrated a trend toward higher MMP-7 mRNA expression levels in NSCLCs with lymph node metastasis. By contrast, our results showed no correlation between MMP-7 and any clinicopathological factor.

We also analyzed the relationship of IGF-1R and MMP-7 expression to post-surgical outcomes. Previously, Merrick *et al* (20) analyzed IGF-1R expression in 184 surgically treated patients with stage I to IV NSCLC. In stage I disease, high IGF-1R expression was associated with significantly shorter survival than low IGF-1R expression. Dziadziuszko *et al* (17) evaluated 189 NSCLCs and showed that the *IGF-1R* gene copy number is of prognostic value; nonetheless, IGF-1R protein expression upon immunohistochemical analysis was not related to survival. Ludovini *et al* (19) reported that IGF-1R protein expression alone was not significantly associated with survival, although high co-expression of both IGF-1R and epidermal growth factor receptor was associated with shorter

Table IV. Subset analysis of overall survival in NSCLC.

	5-year survival rate (%)					
Variables	IGF-1R-high	IGF-1R-low	P-value	MMP-7-high	MMP-7-low	P-value
Gender						
Male	25.9	54.5	0.022	24.0	55.5	0.051
Female	50.0	87.5	0.064	62.2	66.6	0.955
Histological type						
Adenocarcinoma	38.1	68.4	0.016	48.9	59.0	0.608
Squamous cell carcinoma	29.4	55.5	0.586	23.3	54.5	0.478
Pathological stage						
I	53.8	72.8	0.182	61.1	71.4	0.601
II, III	25.0	53.5	0.072	22.2	50.0	0.246
Smoking status						
Smoker	31.0	57.6	0.050	28.8	57.1	0.104
Non-smoker	41.6	77.7	0.087	54.5	62.5	0.913

Table V. Multivariate analysis of overall survival in NSCLC.

Variables	P-value	Hazard ratio	95% confidence interval
Gender	0.016	2.598	1.198-5.638
T-factor	0.007	2.540	1.291-4.997
IGF-1R	0.011	2.322	1.215-4.436

disease-free survival in resected NSCLC. Cappuzzo *et al* (18) concluded that IGF-1R expression does not represent a prognostic factor in resected NSCLC patients.

In the present study, overall survival was significantly poorer in patients with IGF-1R-high tumors than in those with IGF-1R-low tumors, and multivariate analysis showed IGF-1R expression as an independent indicator of poor outcomes. As for MMP-7, Liu *et al* (12) reported that the overall survival rate was significantly lower in patients with MMP-7-positive NSCLC than in those with MMP-7-negative NSCLC. On the other hand, Leinonen *et al* (13) reported that MMP-7 had no prognostic value in NSCLC. Our results showed a trend toward poorer survival in patients with MMP-7-high tumors than in those with MMP-7-low, but the difference fell short of reaching statistical significance.

In conclusion, our results suggest that overexpression of IGF-1R is a useful predictor of lymph node metastasis and recurrence in patients with NSCLC. Overexpression of IGF-1R may thus be an important prognostic factor along with gender and T-factor in patients with NSCLCs.

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