Activation of AXL and Antitumor Effects of a Monoclonal Antibody to AXL in Lung Adenocarcinoma

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Abstract. *Background/Aim: AXL (anexelekto) has been explored as a potential novel therapeutic target for non-small cell lung carcinoma (NSCLC) but its activation status has not been evaluated in NSCLC. Patients and Methods: We first immunolocalized the phosphorylated form of AXL in 112 lung adenocarcinoma cases and subsequently evaluated the anti-neoplastic effects of monoclonal antibody AXL in two lung adenocarcinoma cell lines, PC9 and A549. Results: Phospho-AXL immunoreactivity was detected in 59.8% of adenocarcinoma cases examined and tended correlate significantly with larger tumor size (p=0.08) and with overall survival of the patients (p=0.041). Results of in vitro analysis revealed that the monoclonal antibody to AXL significantly inhibited cell proliferation of PC9 and A549, lung adenocarcinoma cell lines, which was caused by an inhibition of extracellular signal-regulated kinase (ERK) activation. Conclusion: AXL-targeted therapy, possibly through inhibiting ERK activation of carcinoma cells, could confer clinical benefits on patients with lung adenocarcinoma.*

AXL is a member of the receptor tyrosine kinase (RTK) family known as the TAM (TYRO3-AXL-MER) family, including TYRO3 and MER (1, 2). This family is characterized by an extracellular domain which contains immunoglobulin-like and fibronectin type III domains and can be proteolytically-cleaved to release a soluble form (3). Both growth arrest-specific gene 6 (GAS6) and protein S have been identified as the ligand for the TAM family and in particular, GAS6 was reported to have the highest affinity for AXL (4-8). Six different types of AXL activation mechanisms have been reported as follows: (i) ligand-induced dimerization, (ii) ligand-independent homophilic dimerization, (iii) heteromeric dimerization of two different TAM receptors, (iv) heterotypic interaction with a non-TAM receptor, (v) trans-cellular binding of extracellular domains and (vi) transactivation mediated by SRC (9-11). It is also well-known that the typical activation mechanism of RTKs is ligandbinding to the extracellular domain (12). In this case, the binding of ligand GAS6 to AXL induced receptor dimerization, which resulted in autophosphorylation of tyrosine residues in intracellular domains (7). Autophosphorylation further activated its downstream signaling pathways such as AKT and mitogen-activated protein kinase (MAPK) phosphorylation, resulting in induction of cell proliferation, growth and the other cellular functions. However, it is also true that activation of TAM receptors can occur without ligand binding in some tumor types (13-15). As for (ii) and (iii) above, overexpression of AXL and TYRO3 was reported to cause AXL homophilic (13) and AXL–TYRO3 dimerization (14, 15), which subsequently induced its phosphorylation. In contrast, ligand-independent heterotypic dimerization of AXL with interleukin-15-alpha (IL-15α) receptor has been reported in immortalized and primary fibroblasts (iv) (16). IL-15 was also reported to transactivate AXL and cause heterotypical dimerization of AXL and IL-15α receptor. However, AXL was not reported to precipitate other IL receptor subtypes, even when its ligands were present. In addition, a novel mechanism of AXL activation was recently reported by Ruan *et al.*, who demonstrated that vascular endothelial growth factor A (VEGFA)-dependent phosphoinositide 3-kinase (PI3K)/AKT activation was mediated by AXL activation through the phosphorylation of SRC kinase family through interacting with VEGF receptor-3 (VEGFR3) (v) (11). AXL is also known as the major effector of cell survival,

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proliferation and migration in several human malignancies, such as esophageal adenocarcinoma (17), gastric cancer (18), ovarian cancer (19) and lung cancer (20, 21). However, the phosphorylated form of AXL has not been studied in any human malignancy to the best of our knowledge, despite its putative importance in biological behavior.

AXL overexpression in carcinoma cells was reported to be correlated with increased invasiveness (20) and cellular adhesion (22) in non-small cell lung carcinoma (NSCLC), especially in lung adenocarcinoma. Therefore, AXL has been proposed as a novel therapeutic target in NSCLC and AXL-targeted monoclonal antibodies were produced, which did suppress lung adenocarcinoma cell growth (23, 24). In addition, AXL was also reported to play important roles in the development of therapeutic resistance to sulfasalazine, a synthetic non-steroidal anti-inflammatory drug, and epidermal growth factor receptor (EGFR)-targeted therapy in lung adenocarcinoma (25, 26). However, the correlation between AXL functions and tumor cell proliferation or clinical outcome of the patients has not been reported at all in NSCLC. Therefore, in the present study, we first examined whether AXL activation was involved in tumor progression of lung adenocarcinoma in patients or not by evaluating the status of phosphorylated AXL using immunohistochemistry and correlated the findings with clinicopathological features of patients. We further examined the effects of monoclonal antibody to AXL, on whether the antibody blocked the ability of GAS6 to activate AXL or not, using lung adenocarcinoma cell lines.

Patients and Methods

Antibodies and reagents. Antibodies used for immunohistochemistry in the present study were as follows: anti-phospho-AXL (R&D Systems Inc., Minneapolis, MN, USA); anti-Ki67 (DAKO, Glostrup, Denmark). Those used for immunoblotting analysis were as follows: anti-AXL and anti-GAS6 (all from R&D Systems); anti-p44/42 MAPK (ERK) and anti-phospho-ERK (both from Cell Signaling Technology, Inc., Beverly, MA); anti-β-actin (Sigma Aldrich Co., Ltd., St Louis, MO, USA). β-Actin was used as internal standard in this study.

Monoclonal antibody to AXL was produced using the hybridoma method at Chugai Pharmaceutical Co., Ltd. (Shizuoka, Japan) (US patent 20120121587). In brief, mouse-derived antibodies to AXL which were bound to the extracellular domain of human AXL were selected and humanized anti-AXL antibodies were subsequently obtained. These anti-AXL antibodies were demonstrated to have higher inhibitory activities on ligand-dependent AXL phosphorylation using cell lines Calu-1 and MDA-MB-231, derived from human lung squamous cell and breast carcinoma, respectively. The antitumor activities of these antibodies were further confirmed both *in vitro* (human colorectal cancer cell line HCT-116) and *in vivo* (human pancreatic adenocarcinoma cell line PANC-1 xenograft model).

Lung adenocarcinoma cases. A total of 112 lung adenocarcinoma specimens, which were randomly collected from the pathology files, were diagnosed between 1993 and 2004 and retrieved from the pathology files of the Sendai Kosei Hospital and Tohoku University Hospital (both in Sendai, Japan). Clinicopathological characteristics of the cases examined in this study are summarized in Table I. Tissues were fixed with 10% formalin and embedded in paraffin. Research protocols for this study were approved by the Ethics Committee at the Tohoku University School of Medicine (2009-380).

Table I. *Clinicopathological features of 112 lung adenocarcinoma cases examined in this study.*

Clinicopathological features	n=112
Age (years)*	66.6±9.2
Gender, n	
Male	62
Female	50
Tumor size (mm)	28.6±14.1
Stage, n	
I	79
II	12
III	21
Ki-67 LI (%)*	16.4±12.2

*Data are presented as mean±SEM. LI: Labeling index.

Immunohistochemistry. We immunostained the sections using an EnVision+ kit (DAKO; phospho-AXL) and Histofine kit (Ki67; Nichirei Co., Ltd, Tokyo, Japan). Antigen retrieval procedure was not employed in immunostaining for phospho-AXL and that for Ki-67 was performed by heating the slides using an autoclave at 121˚C for 5 min in citrate buffer. The dilution of the antibodies was as follows: phospho-AXL, 1/200; Ki67, 1/100. Phospho-AXL immunoreactivity was detected only in the cytoplasm of carcinoma cells, but not in alveolar and bronchial epithelial cells of adjacent non-neoplastic lung tissues and stromal cells including fibroblasts, inflammatory cells, endothelial cells and others. The status of immunoreactivity was tentatively classified into the following three groups: negative, weakly-positive and markedly positive according to Rettew *et al*. (27). The percentage of Ki-67 immunoreactivity, *i.e*. labeling index (LI), was determined by counting 1,000 carcinoma cells with nuclear immunoreactivity of Ki-67 in hot spots in adenocarcinoma specimens. All scoring was performed independently by two of the authors (SI and YM). When inter-observer differences were more than 5%, the immunostained slides were simultaneously evaluated together by the two authors above using a multi-headed light microscopy and the scores were obtained. When inter-observer differences were less than 5%, the score of the cases examined was the mean of the two scores.

Cell culture. A549, NCI-H1975 and NCI-H23 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), PC9 was from Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan) and PC3 was from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). Cells were incubated with RPMI-1640 medium (Sigma Aldrich Co., Ltd.) containing 10% of fetal bovine serum (FBS; Nichirei Co.) in a humidified incubator in 5% CO_2 at 37˚C.

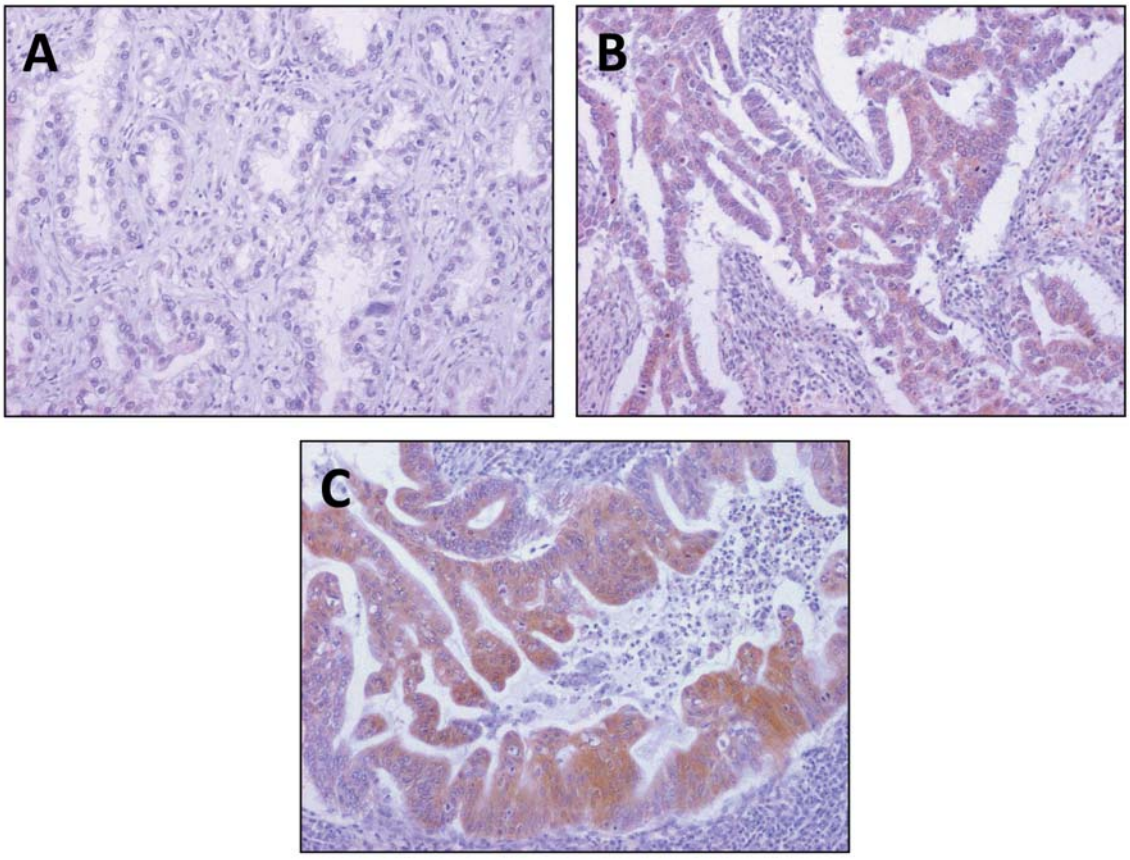

Figure 1. *Representative illustrations of phosphorylated AXL in NSCLC tissues. Phospho-Axl immunoreactivity was evaluated and classified into three groups as follows: negative group (A); weakly-positive group (B); markedly-positive group (C). Original magnification, ×100. D: Summary of overall survival analysis in 112 NSCLC cases examined according to the status of phospho-Axl immunoreactivity. Survival curves were obtained using Kaplan-Meier method and the p-Value was evaluated using log-rank test.*

Cell proliferation assay. Cells were seeded in 96-well plates and treated in a concentration-dependent manner (0, 1.0, 2.0 and 5.0 ng/ml) with monoclonal antibody to AXL for 72 h. After the treatment, the cell number was counted using WST-8 Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). The cell number was evaluated as follows: (average absorbance of treated cells/average absorbance of control cells)×100 (%).

Immunoblotting analysis. Total proteins were extracted from treated cells using Mammalian Protein Extraction Reagent containing Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (all from Pierce Biotechnology, Rockford, IL, USA). Those concentrations were measured using the Protein Assay Kit (Wako Pure Chemical Industries). After electrophoresis, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK) and finally detected using ECL-plus detection reagents (GE Healthcare).

Statistical analysis. Statistical analysis was carried-out using StatMate IV (ATMS Co., Ltd., Tokyo, Japan). Univariate and multivariate analyses were performed using StatView 5.0 J software (SAS Institute Inc., Cary, NC, USA). A value of $p<0.05$ was considered to represent a significant difference.

Results

Phospho-AXL in NSCLC specimens is correlated with poor prognosis. We first examined the status of phosphorylated form of AXL in 112 lung adenocarcinoma cases. Representative immunoreactivity of three different groups of phospho-AXL immunoreactivity is illustrated in Figure 1. Phospho-AXL immunoreactivity was detected only in the cytoplasm of adenocarcinoma cells but not in normal lung tissues and stromal cells in cancer tissues. Results of this analysis is summarized as follows: negative, 40.2% (45 /112); weakly positive, 38.4% (43 /112); markedly positive, 21.4% (24/112).

The status of phospho-AXL immunoreactivity tended correlate with larger tumor size ($p=0.08$, Table II). There were no significant correlations between the status of phospho-AXL immunoreactivity and Ki-67 LI ($p=0.59$) of adenocarcinoma cells, but the Ki67 LI in the markedly positive group was the highest among the three groups (Table II). In addition, the status of phospho-AXL immunoreactivity in carcinoma cells

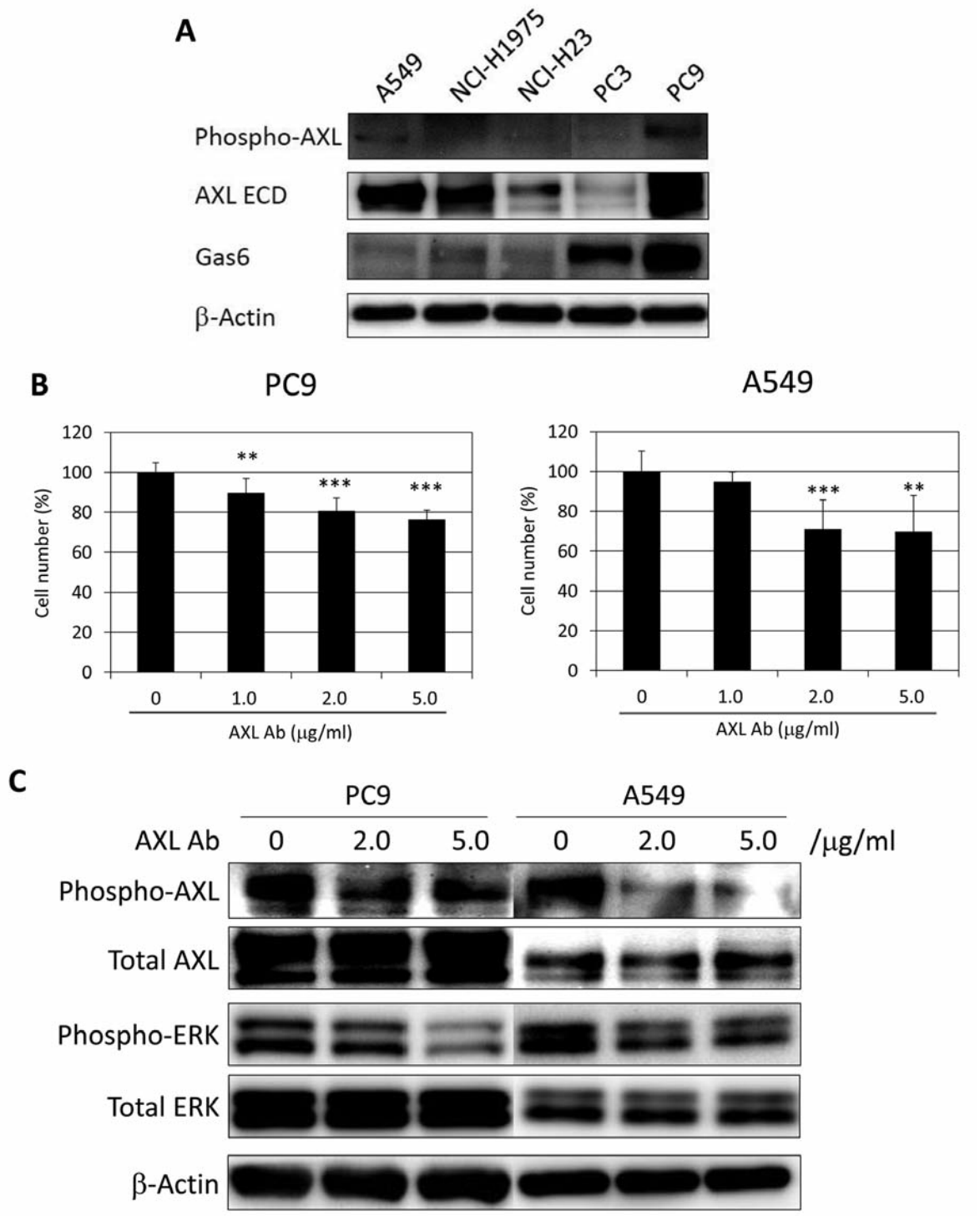

Figure 2. *Antitumor effects of monoclonal antibody to AXL in lung adenocarcinoma cells. A: Expression of Axl, GAS6 and phospho-Axl in lung adenocarcinoma cell lines. The expression of Axl and Gas6 in five types of lung adenocarcinoma cell lines (A549, NCI-H1975, NCI-H23, PC3 and PC9) was evaluated at the protein level. B: Proliferation assay when lung adenocarcinoma cells were treated with monoclonal antibody to AXL. NSCLC cell lines PC9 and A549 were treated with monoclonal antibody against AXL in a concentration-dependent manner (0, 1.0, 2.0 and 5.0 μg/mL) for 72 h and the effects on cell proliferation were evaluated (n=6). p-Values were evaluated using Student's t-test. **p<0.01, ***p<0.001. C: Effects of monoclonal antibody to AXL on the activation of Axl and its signal transduction in lung adenocarcinoma cell lines. Monoclonal antibody to AXL was added to PC9 and A549 cells at 0, 2.0 and 5.0 μg/ml for 1 h and the phosphorylation of AXL and their downstream MEK–ERK pathway was evaluated. AXL Ab, monoclonal antibody to AXL.*

was significantly associated with poor clinical outcome of patients with adenocarcinoma (p=0.041, Figure 1D).

Table III summarizes the results of the univariate and multivariate analyses. The presence of phospho-AXL immunoreactivity significantly correlated with adverse clinical outcome of patients examined in both univariate and multivariate analyses (p=0.009 and 0.01, respectively), but other factors did not reach a statistically significant level (Table III).

Antitumor effects of monoclonal antibody to AXL in PC9 and A549 cells. In order to select which lung adenocarcinoma cell lines to use in this study, the basal expression of AXL and its ligand GAS6was evaluated at the protein level in five types of cell lines (A549, NCI-H23, NCI-H1975, PC3 and PC9). The results indicated that AXL expression was detected at higher levels in PC9, A549 and NCI-H1975 cells (Figure 2A). Among these cell lines, higher expression of GAS6 was indicated only in PC9. Although AXL was less detected in PC3 cells, GAS6 was highly expressed. Both AXL and GAS6 were less expressed in NCI-H23 cells. Based upon these results, we selected two cell lines: PC9, which was considered to activate AXL by GAS6 in an autocrine manner, and A549, which was considered to activate AXL in a ligand-independent manner.

We subsequently evaluated the antitumor effects of monoclonal antibody to AXL, which was demonstrated to inhibit the binding of GAS6 to AXL extracellular domain and to subsequently suppress the activation of AXL (US patent 20120121587), in lung adenocarcinoma cell lines PC9 and A549. The monoclonal antibody significantly inhibited cell proliferation in both cell lines in a concentration-dependent manner (Figure 2B). Results of immunoblotting demonstrated that AXL was auto-phosphorylated in both PC9 and A549 cells at control levels and anti-AXL also inhibited the activation of AXL and ERK (Figure 2C).

Table II. *Association between phospho-AXL and clinicopathological factors.*

	Negative	Weakly positive	Markedly positive	p-Value
	n=45	n=43	n=24	
Age (years)*	66.0±9.6	66.9±8.4	67.0±8.4	0.86
Gender, n				0.55
Male	21	30	11	
Female	24	13	13	
Tumor size (mm)*	28.0±14.2	26.3±12.7	33.8±12.1	0.08
Stage, n				0.24
I	33	32	14	
II	2	6	4	
III	10	5	6	
Ki-67 LI (%)	15.3±10.5	15.7±12.7	18.7±12.2	0.59

*Data are presented as mean±SEM. LI: Labeling index. p-Values of <0.05 were considered statistically significant.

Table III. *Results of univariate and multivariate analyses of overall survival of the patients in this study.*

Variable	Overall survival		HR (95% CI)
	Univariate	Multivariate	
	p-Value	p-Value	
Phospho-Axl immunoreactivity (Positive/Negative)	0.009	0.01	2.3 (1.2-4.1)
TNM Stage (II and III/I)	0.13		
Ki-67 LI (≥10%/<10%)	0.59		
Tumor size (≥30 mm/<30 mm)	0.8		
Gender (Male/Female)	0.98		

HR, Hazard ratio; CI, confidence interval. p-Values of <0.05 were considered statistically significant.

Discussion

Receptor tyrosine kinases are activated by their specific ligands or in a ligand-independent manner, and promote several biological functions through activating downstream signaling pathways such as PI3K-AKT and MEK–ERK pathways (27, 28). Therefore, two types of agents targeting against RTKs have been developed (29). The first is the monoclonal antibody for blocking ligand binding to receptors, which also results in antibody-dependent cytotoxic reaction by immune cells, and the other is specific small molecule inhibitors of tyrosine kinase to suppress the activity of autophosphorylation sites (29). AXL has been reported to play important roles in tumor progression in several human malignancies (23, 30) and both AXL-targeted monoclonal antibody and its tyrosine kinase inhibitor have been developed (10). However, the status and role of AXL activation has not been examined in any type of human malignancy.

Therefore, in the present study, we first evaluated the status of phosphorylated form of AXL in lung adenocarcinoma cases using immunohistochemistry with the recently developed specific monoclonal antibody (31) and further evaluated the correlation between results of immunohistochemistry and clinicopathological features of lung adenocarcinoma cases examined. In approximately 60% of lung adenocarcinoma cases examined, cytoplasmic phospho-AXL immunoreactivity was associated with carcinoma cells but not adjacent non-neoplastic lung alveolar or bronchial cells, and overactivation of AXL or cases demonstrating marked phospho-AXL immunoreactivity tended to be

associated with larger tumor size and high cell proliferation of tumor cells, as well as, significantly, with poor prognosis of the patients. These findings also indicated that AXL overactivation did induce cell proliferation of lung adenocarcinoma cells and subsequently resulted in adverse clinical outcome of the patients but this awaits further investigations for clarification.

We then studied whether monoclonal antibody to AXL inhibited AXL activation in lung adenocarcinoma cell lines PC9 and A549 or not. In these cell lines, AXL is phosphorylated at control levels without adding exogenous GAS6. In PC9, GAS6 was expressed at high levels among lung adenocarcinomas examined and thus AXL was activated in an autocrine loop. Monoclonal antibody against AXL was thus considered to inhibit AXL activation by GAS6-binding to AXL extracellular domain in PC9 cells. In A549 cells, GAS6 was less expressed but AXL was activated, suggesting that overexpression of AXL induced dimerization and auto-phosphorylation, as previously reported (13). This reagent was considered to inhibit the phosphorylation of AXL by binding to GAS6-binding region through blocking of AXL dimerization in A549 cells.

Monoclonal antibody against AXL reduced the expression of phospho-ERK but not phospho-AKT (data not shown), indicating that AXL predominantly induced cell proliferation through the MEK–ERK pathway rather than the PI3K–AKT pathway in these cell lines. The AXL downstream signaling pathway has been studied in cell lines derived from various human normal cells and malignancies (9). GAS6-related cell survival mediated by PI3K–AKT pathway has been reported in human oligodendrocytes (32), endothelial cells (33), vascular smooth muscle cells (34) and lens epithelial cells (35). However, GAS6-induced ERK activation has been also reported in cardiac fibroblasts and prostate carcinoma cells resulting in promotion of cell proliferation (36, 37). Therefore, AXL function in these cells, mediated by the MEK–ERK pathway as demonstrated our results, is considered a specific mechanism for in lung adenocarcinoma cells.

Phosphorylation/activation is usually considered crucial to RTKs, including AXL, in order for them to be fully-functional in cells. However, the status of phosphorylated AXL in human malignant tissues has not been previously evaluated. In the present study, we demonstrated antitumor effects only in NSCLC cell lines which secreted GAS6 in an autocrine fashion. As described above, the dimerization and activation of AXL without GAS6 have been reported (9-11). These results also suggest that the antibody against AXL may be considered less effective on AXL activation by extracellularly-derived GAS6. The status of AXL phosphorylation was not correlated with proliferative activities of carcinoma cells, but these results indicate antitumor effects of monoclonal antibody to AXL. Further investigations are required to unravel the mechanism of AXL activation and develop AXL-targeted therapy in individual cases of lung adenocarcinoma.

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