three separate levels; progressive disease: at least 20% increase in the sum of the pleural thickness at three separate levels or the appearance of one or more new lesions; stable disease: neither sufficient shrinkage to quality for partial response nor a sufficient increase to qualify for progressive disease. The cases diagnosed before the establishment of the RECIST criteria were re-evaluated by two independent reviewers. The response rate was defined as the sum of complete responses and partial responses. A resection was defined as complete (R0) if all gross disease was removed and if all surgical margins were free of the tumor. Incomplete resection (R1) meant that all cancer tissue except pleural dissemination was removed. An exploratory thoracotomy meant that the tumor was judged to be unresectable during the operation and no resection or only a biopsy was performed.

Statistical Analysis

The survival was calculated from the date of the initial treatment until death due to any cause or the last follow-up (censored). The survival curve was made using the Kaplan–Meier method, and statistical differences between survival curves were examined using the log-rank test. The confidence intervals at 5 and 10 years on the survival curve were calculated based on the cumulative hazard function. The impact of the WHO classification on tumor response to treatment was assessed using the χ^2 test. All data were analyzed using Abacus Concepts Survival Tools for StatView (Abacus Concepts, Berkeley, CA, USA).

Results

Treatment

Figure 1 shows a schematic illustration of the treatment strategy. The treatment strategy for advanced thymoma has varied over time. Surgical resection was performed as the primary treatment throughout the study period, when the tumor was judged to be resectable or marginally resectable. Radiotherapy was primarily selected in the period from the 1970s to mid-1980s (patients 1, 2, 3, 5, 6, and 8) for patients with unresectable tumors or those who underwent an exploratory thoracotomy, and some patients concurrently received chemotherapy (patients 4 and 7). Chemotherapy with the ADOC regimen was used instead of radiotherapy in the mid-1980s (patients 9, 10, and 12). However, radiotherapy was performed for elderly patients (patients 16 and 19). Concurrent chemoradiotherapy using cisplatin or carboplatin has been recently introduced (patients 17, 21, and 22).

Surgery was performed in 10 patients. A complete resection (R0) was performed in 2 patients without adjuvant therapy. An incomplete resection (R1) was performed in 2 patients, due to pleural dissemination followed by chemotherapy in 1 patient. An exploratory thoracotomy was performed in 6 patients. Eighteen patients were treated, including 6 patients who underwent an exploratory thoracotomy; 8 patients were initially treated with radiotherapy, 5 patients with chemotherapy, and 5 patients with chemoradiotherapy. Ten patients were treated with subsequent therapy, including chemotherapy in 3, radiotherapy in 4, and surgery in 3. Subsequent salvage surgery resulted in an R1 resection in 1 patient and an exploratory thoracotomy in 2 patients.

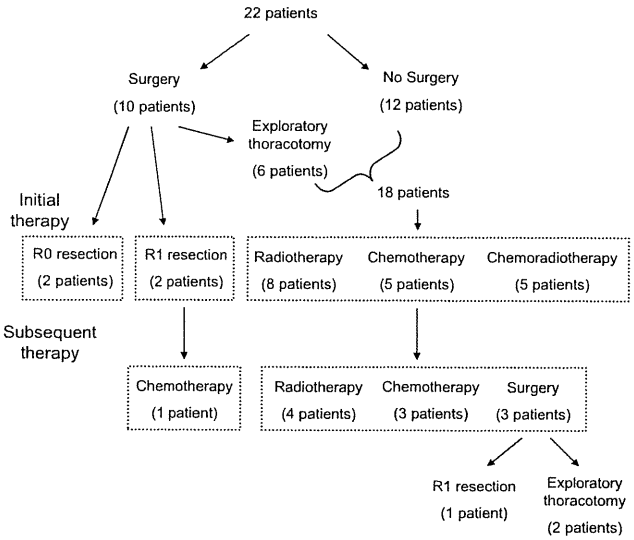

Fig. 1. Schematic illustration of the treatment protocol of the 22 patients with stage III–IV thymomas

Treatment Response

The responses to the initial therapies are summarized in Table 3. The total response rate was 55.5% in the 18 patients with unresectable tumors. The response rate to the initial therapy of 9 patients with WHO type A-B2 thymomas was significantly higher than that of 9 patients with type B3 tumor (100% vs 11.1%, respectively; $P = 0.0001$). The response rate to each therapy was 60%, 50%, and 60% for chemotherapy, radiotherapy, and chemoradiotherapy, respectively.

Recurrence

Tumor progression after the initial and subsequent therapy was confirmed in 12 patients, with a median progression-free interval of 10 months (range, 5–67 months). The patterns of tumor progression were intrathoracic in 8 patients, distant metastasis in 3 patients, and both

Table 3. Response to initial therapy

	Therapy							
	Chemotherapy		Radiotherapy		Chemoradiotherapy		Total	
WHO type	*n*	RR, %	*n*	RR, %	*n*	RR, %	*n*	RR, %
A–B2	3	100	3	100	3	100	9	100
B3	2	0	5	20	2	0	18	11.1
Total	5	60	8	50	5	60	18	55.5

RR, response rate

intrathoracic and distant metastasis in 1 patient. One of the two patients who underwent R0 resection had a pleural recurrence 13 months after surgery. The patient underwent extrapleural pneumonectomy (R0 resection) and is now alive with no disease 145 months after the first surgery. The other patient with R0 resection is alive with no recurrence 132 months after surgery.

Survival

At a median follow-up of 61.9 months (range, 1.7–168.7 months), 9 patients were still alive (2 were disease-free) whereas 13 patients died including two nontumor-related deaths. One patient who had type B3 tumor with pure red cell aplasia died of pneumonia 25 months after the initial therapy. The other patient, who also had a type B3 tumor but without autoimmune disease, died of cardiac failure 5 months after the initial therapy. The median survival time was 73 months. The overall survival rates at 5 and 10 years for all patients were 64.9% (95% confidence interval [CI] 39.7%–81.6%) and 43.2% (95% CI 19.2%–65.3%), respectively (Fig. 2). The survival curves of 18 patients who had stage III or IVa disease without R0 resection in relation to the WHO classification are shown in Fig. 3. The 5-year survival rates were 88.9% (95% CI 43.3%–98.4%) for 10 patients with WHO type A-B2 thymomas (excluding 2 patients with R0 resection) and 33.3% (95% CI 9.0%–61.1%) for 8 patients with WHO type B3 tumors (excluding 2 patients with stage IVb disease), which were significantly different ($P = 0.01$). The Masaoka staging system showed the 5- and 10-year survival rates to be 50.0% (95% CI 15.2%–77.5%) and 33.3% (95% CI 5.6%–65.8%) for 8 patients with stage III tumors, and 74.0% (95% CI 38.2%–91.0%) and 37.0% (95% CI 9.1%–66.2%) for 14 patients with stage IV tumors, which were not significantly different. The association of autoimmune diseases was not correlated with the survival.

Discussion

Thymomas are often chemotherapy-sensitive tumors, and therefore chemotherapy has been adopted in patients

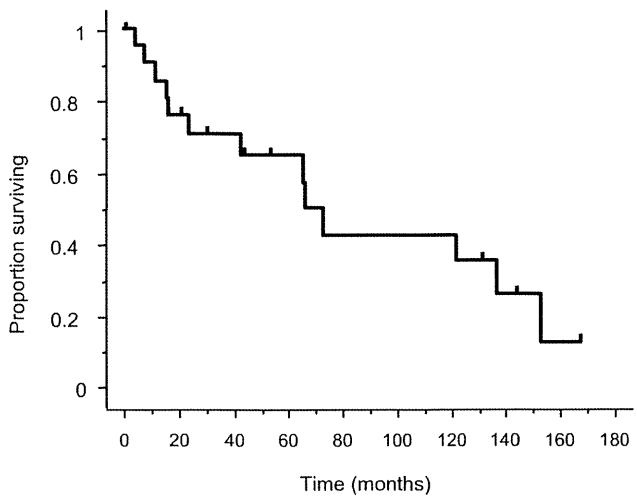

Fig. 2. Overall survival of 22 patients with stage III–IV thymomas. The median survival time was 73 months. The overall survival was 64.9% at 5 years and 43.2% at 10 years

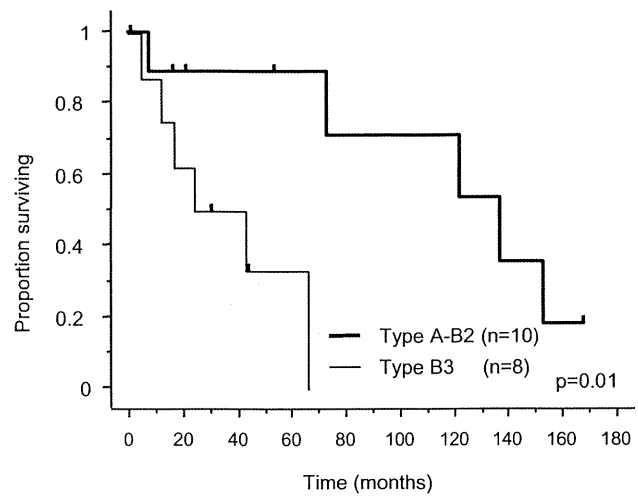

Fig. 3. Survival of 18 patients with stage III or IVa disease without R0 resection according to the World Health Organization histological classification. The 5-year survival rate was 88.9% for 10 patients with type A-B2 and 33.3% for 8 patients with type B3, which showed a significant difference ($P = 0.01$)

with advanced thymomas. Frequently applied agents include cisplatin-based regimens consisting of ADOC, cisplatin, doxorubicin, and cyclophosphamide (PAC), or etoposide, ifosfamide, and cisplatin (VIP). The clinical response rates for unresectable or recurrent thymomas range between 32% and 92%, including 10%–43% of complete response.[25] One of the reasons for this wide variation in response rate could be related to the variation in the histology associated with the WHO histological classification. The current study showed that type A-B2 thymoma showed a better response rate to chemotherapy and/or radiotherapy than type B3 thymoma regardless of the treatment modality.

The WHO classification of thymoma is based on morphological features, such as the presence or absence of immature lymphoid components and the degree of cytological atypia. Types AB, B1, and B2 thymomas have a structure similar to that of the cortex in a normal thymus.[26] This classification system is a significant prognostic factor for thymoma. Park et al. reported that type B3 and C tumors are associated with poorer survival than the other types of thymoma.[16] Lucchi et al. reported that type B3 thymoma has a more aggressive clinical behavior with a significantly worse prognosis than the other thymomas; however, no correlation is observed between the objective response rate and histological type of disease.[23] Onuki et al. reported that both type B1 and B2 thymomas were significantly better than type B3 thymomas in both radiological and histological radioresponse among 21 patients with stage III thymomas who underwent preoperative radiotherapy.27 They also showed the reduction ratio to be significantly lower in type B3 thymomas than in type B1 or B2 thymomas, and type B3 thymomas did not show obvious histological changes after radiation. Onuki et al. speculated that the difference in the histological changes after radiation between type B2 and B3 thymomas may thus be attributable to the difference in the radioresponse of their epithelial tumor cells.

A genetic analysis of thymic epithelial tumors revealed a relationship between genetic instability and the WHO classification of thymoma. An examination that utilized microsatellite analysis revealed that the incidence of genetic imbalances was more frequent in type B2 and B3 thymomas and thymic carcinoma than in the other types of thymomas.[28] Zettl et al. reported that studies using comparative genomic hybridization and fluorescence in situ hybridization showed that genomic aberrations occur frequently in type B3 thymomas.[29] Another examination of gene amplification of epithelial growth factor receptor (EGFR) revealed that the average number of EGFR gene signals per cell was higher in type B3 thymomas than in the other types.[30] This genetic instability of type B3 thymoma might be related to therapeutic response.

Multimodality treatment including induction chemotherapy for locally advanced thymoma has been recently introduced, and favorable outcomes have been reported.[5,8–15] The chemotherapeutic regimens administered to date have been diverse and the response rates in these induction settings range from 67% to 100%, while the complete resection rates are reported to be around 70%.[5,8–10,13] Wright et al. recently reported the results from 10 patients with advanced thymic tumors treated with preoperative concurrent chemoradiation followed by resection.[15] The response rate was 40% and the complete resection rate was 80%. The response rate seemed to be low in comparison to other reports; however, 7 of 10 patients had type B3 thymomas and 1 patient had type C thymoma. Half of the patients who were treated with chemotherapy and/or radiotherapy in the current series had type B3. Although the response rate was 100% in 9 patients with type A-B2 thymoma, the total response rate in 18 patients was 55.5%. It should be noted that response rate could be affected by the ratio of the patients who had type B3 thymoma.

In conclusion, type B3 thymoma showed a lower response rate to treatments and thus a shorter survival. Therefore, the WHO classification is considered to be an effective predictive factor for therapeutic response in advanced thymoma.

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Conflict of Interest Statement. The authors have no conflict of interest or financial support to declare.

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Hepatocyte Growth Factor Expression in *EGFR* Mutant Lung Cancer with Intrinsic and Acquired Resistance to Tyrosine Kinase Inhibitors in a Japanese Cohort

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Introduction: This study was performed to determine the incidence rates of resistance factors, i.e., high-level hepatocyte growth factor (HGF) expression, epidermal growth factor receptor (EGFR) T790M secondary mutation, and *MET* amplification, in tumors with intrinsic and acquired EGFR tyrosine kinase inhibitor (TKI) resistance in *EGFR* mutant lung cancer.

Methods: Ninety-seven specimens from 93 *EGFR* mutant lung cancer patients (23 tumors with acquired resistance from 20 patients, 45 tumors with intrinsic resistance from 44 patients [nonresponders], 29 sensitive tumors from 29 patients) from 11 institutes in Japan were analyzed. HGF expression, *EGFR* T790M secondary mutation, and *MET* amplification were determined by immunohistochemistry, cycleave real-time polymerase chain reaction, and fluorescence in situ hybridization, respectively.

Results: High-level HGF expression, *EGFR* T790M secondary mutation, and *MET* amplification were detected in 61, 52, and 9% of tumors with acquired resistance, respectively. High-level HGF expression was detected in 29% of tumors with intrinsic resistance (nonresponders), whereas *EGFR* T790M secondary mutation and *MET* amplification were detected in 0 and 4%, respectively. HGF expression was significantly higher in tumors with acquired resistance than in sensitive tumors ($p < 0.001$, Student's t test). Fifty percent of tumors with acquired resistance showed simultaneous HGF expression with *EGFR* T790M secondary mutation and *MET* amplification.

Conclusions: High-level HGF expression was detected more frequently than *EGFR* T790M secondary mutation or *MET* amplification in tumors with intrinsic and acquired EGFR-TKI resistance in *EGFR* mutant lung cancer in Japanese patients. These observations provide a rationale for targeting HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer.

Key Words: EGFR-TKI, EGFR mutation, HGF, Acquired resistance, Intrinsic resistance.

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Epidermal growth factor receptor (EGFR)-activating mutations, in-frame deletion in exon 19, and L858 point mutation in exon 21 are selectively expressed in a population with lung cancer.[1,2] *EGFR*-activating mutations are detected considerably more frequently in nonsmokers, females, adenocarcinomas, and patients from East Asia, including Japan.[3–5] The reversible EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib show dramatic therapeutic efficacy, response rates of 70 to 80%, and significant prolongation of progression-free survival (PFS) compared

with standard first-line cytotoxic chemotherapy in patients with *EGFR* mutant lung cancer.[6–9] However, patients almost always develop acquired resistance to EGFR-TKIs after varying periods.[6,9,10] In addition, 20 to 30% of patients with *EGFR*-activating mutations show intrinsic resistance to EGFR-TKIs.[4] Therefore, intrinsic and acquired resistance to EGFR-TKIs are major problems in management of *EGFR* mutant lung cancer.

Two genetically conferred mechanisms—*EGFR* T790M secondary mutation (T790M secondary mutation)[11,12] and *MET* gene amplification[13]—induce acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer. In addition, we recently demonstrated the occurrence of hepatocyte growth factor (HGF)-induced resistance.[14] HGF, a ligand of MET,[15] induces EGFR-TKI resistance by activating MET, which restores phosphorylation of downstream MAPK-ERK1/2 and PI3K-Akt pathways,[14] using Gab1 as an adaptor.[16] HGF may be involved in both intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer.[14]

T790M secondary mutation, *MET* amplification, and high-level HGF expression were detected in clinical specimens from *EGFR* mutant lung cancer patients who acquired resistance to EGFR-TKIs,[11–14,16–18] indicating the clinical relevance of all three resistance mechanisms in lung cancer. Although the number of cases in each study was limited (<30 cases/study), probably because of low availability of biopsy specimens from resistant tumors, *EGFR* T790M secondary mutation and *MET* amplification were estimated to have occurrence rates of 50%[11,12,17,19] and up to 20%,[13,16,17] respectively, in patients showing acquired resistance to EGFR-TKIs. Nevertheless, the incidence of HGF-induced resistance has not been determined. In addition, the incidence rates of these three resistance factors in intrinsic resistance (nonresponders) are unknown.

Here, we performed a large-scale study in 23 tumors with acquired resistance from 20 patients, 45 tumors with intrinsic resistance from 44 patients (nonresponders), and 29 sensitive tumors from 29 patients to determine the incidences of the three resistance factors not only in acquired resistance but also in intrinsic resistance (nonresponders) to EGFR-TKIs in Japanese patients with *EGFR* mutant lung cancer.

MATERIALS AND METHODS

Patient details are described in the Supplementary information (http://links.lww.com/JTO/A197).

Definition of Sensitivity to EGFR TKI

Here, tumors with *EGFR* mutation known to be associated with drug sensitivity (i.e., G719X, exon 19 deletion, and L858R) were obtained from patients before or after treatment with a single EGFR-TKI.[9]

Sensitive tumors were defined as those obtained from patients whose tumors showed a decrease in diameter of at least 30% (either documented partial response or complete response) associated with EGFR-TKI treatment in imaging studies (Response Evaluation Criteria in Solid Tumors [RECIST] version 1.0). Tumor specimens were obtained before EGFR-TKI treatment.

Tumors with acquired resistance were defined as described previously.[9] Briefly, cases showing objective clinical benefit from treatment with an EGFR TKI as defined by either documented partial or complete response (RECIST) or significant and durable (>6 months) clinical benefit (stable disease as defined by RECIST) and systemic progression of disease (RECIST), while on continuous treatment with gefitinib or erlotinib within the last 30 days were defined as showing acquired resistance. Tumor specimens were obtained after systemic progression of disease.

As intrinsic resistance (nonresponders) has not been clearly defined, tumors without response to treatment with an EGFR TKI, i.e., either documented stable disease or progressive disease (RECIST), were defined as showing intrinsic resistance (nonresponders). Tumor specimens were obtained either before or after EGFR-TKI treatment.

Patients

Ninety-seven tumor specimens with EGFR mutations were obtained from 93 lung cancer patients, all of whom provided written informed consent, at 11 institutes in Japan. This study was approved by the Institutional Review Boards of each institute.

Patients' characteristics are shown in Table 1. Eighty-seven patients had adenocarcinomas, one had large cell carcinoma, two had squamous cell carcinoma, two had adenosquamous carcinoma, and one had undifferentiated non-small cell carcinoma. As the first EGFR-TKI, gefitinib and erlotinib were given to 82 and 10 patients, respectively, and the dual inhibitor of EGFR and VEGFR2, vandetanib,[20] was given to 1 patient.

Exon 19 deletion and L858R point mutation in exon 21 of *EGFR* were detected in 40 and 57 of the 97 tumors, respectively (Table 1). Two of these tumors had both exon 19 deletion and L858R point mutation. Two tumors without exon 19 deletion or L858R had G719X. Twenty-three tumors with acquired resistance were obtained from 20 patients after EGFR-TKI treatment. Forty-five tumors with intrinsic resistance (nonresponders) were obtained from 44 patients either before (41 tumors from 41 patients) or after (four tumors from three patients) EGFR-TKI treatment. Twenty-nine sensitive tumors were obtained from 29 patients before EGFR-TKI treatment.

Immunohistochemistry for HGF

Immunohistochemical staining was conducted on formalin-fixed, paraffin-embedded tissue sections (4 μm thick) of tumor specimens with microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0). We used rabbit polyclonal antibody against HGF-α (IBL, Gunma, Japan) at 1:20 dilution as a primary antibody and EnVision/HRP Polymer Reagent (Dako, Glostrup, Denmark) and DAB (3,3'-diaminobenzidine tetrahydrochloride) Liquid (Dako) for detection.

Evaluation of HGF Expression

The percentages of cancer cells with positive cytoplasmic and/or membrane HGF immunoreactivity were evaluated (0 to 100%), and the modal intensity of the positively staining cells on a scale ranged from 0 to 3+ (0, complete

TABLE 1. Patient Characteristics

Number of Patients	Acquired Resistance (n = 20)	Intrinsic Resistance (n = 44)	Sensitive (n = 29)	Total (n = 93)
Age				
Median	59.5	65.5	65	64
Range	32–85	34–76	42–86	32–86
Gender				
Male	6	26	10	42
Female	14	18	19	51
Smoking history				
Former/current Smoker	3	21	11	35
Never smoker	17	23	18	58
Histological type				
Adeno	19	39	29	87
Large cell	0	1	0	1
Squamous cell	0	2	0	2
Undifferentiated non-small cell carcinoma, or adenosquamous	1	2	0	3
EGFR-TKI treatment				
Gefitinib	19	36	27	82
Erlotinib	1	7	2	10
Vandetanib	0	1	0	1
Number of Tumors	n = 23	n = 45	n = 29	n = 97
EGFR mutation status				
Exon 19 deletion	12	14[a]	14[a]	40
L858R	11	30	16	57
G719X	0	2	0	2

[a] One patient's tumor had both exon 19 deletion and L858R point mutation.

absence of staining; 1+, weaker staining than normal bronchial epithelium; 2+, similar staining to normal bronchial epithelium; and 3+, clearly more intense staining than normal bronchial epithelium) (Supplementary Figure 1, http://links.lww.com/JTO/A197). The percentage and intensity were multiplied to give a scoring index (H score) ranging from 0 to 300, according to a previously reported method with minor modifications.[16] Turke et al.[16] reported that HGF expression was significantly higher in specimens with acquired resistance (mean ± SD: 205 ± 106) compared with pretreatment (126 ± 112). On additional evaluation with specimens showing acquired resistance from patients whose tumors were obtained only after acquiring EGFR-TKI resistance, HGF expression was similar (176 ± 126) to that of specimens with acquired resistance in patients with paired tumor specimens; they concluded that these findings with clinical specimens supported the suggestion that HGF mediated resistance to EGFR-TKIs. Therefore, we defined high-level HGF expression as H score ≥200 in this study. Evaluation was performed independently by two investigators (KT and MN) blinded to individual clinical information.

Cycleave Real-Time Polymerase Chain Reaction Assay for T790M Mutation

Details of the cycleave real-time polymerase chain reaction (PCR) assay have been described previously.[21] Briefly, tumor cell-rich areas in hematoxylin and eosin-stained sections were marked under a microscope, and tissues were scratched from the area of another deparaffinized unstained section. Pieces of the scratched tissue were incubated with 1× PCR buffer containing 100 μg/mL proteinase K for 1 hour at 54°C. After heat inactivation at 95°C for 3 minutes, the solution was used directly as the template DNA for the assay. Then, exon 20 of the *EGFR* gene was amplified by real-time quantitative PCR assay on a SmartCycler (Cepheid, Sunnyvale, CA) using Cycleave PCR Core kits (TaKaRa Co. Ltd., Ohtsu, Japan) with a T790M-specific cycling probe and a wild-type cycling probe. This assay detected as few as 5% cancer cells with T790M mutation in a background of cells with wild-type T790M in *EGFR*.

MET Amplification

Formalin-fixed, paraffin-embedded tissue sections (4 μm thick) were subjected to dual-color fluorescence in situ hybridization using a MET/CEP7 probe cocktail (Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's instructions. Staining was evaluated as reported previously.[22,23]

Statistical Analysis

Statistical significance was determined by Student's t test. All statistical analyses were performed using GraphPad

TABLE 2. Expression of HGF, T790M Secondary Mutation, and *MET* Amplification in EGFR-TKI-Resistant Tumors Obtained from *EGFR* Mutant Lung Cancer Patients

	Acquired Resistance (n = 23)	Intrinsic Resistance (n = 45)	Sensitive (n = 29)
High-level HGF expression	14 (61%)	13[a] (29%)	3[b] (10%)
EGFR T790M secondary mutation	12 (52%)	0	0
MET amplification	2 (9%)	2 (4%)	0

[a] High-level HGF expression was detected in the stroma in two patients.
[b] High-level HGF expression was detected in the stroma in one patient.

Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA). All tests were two sided, and $p < 0.05$ was taken to indicate statistical significance.

RESULTS

HGF Expression, T790M Secondary Mutation, and MET Amplification in Tumors with Acquired Resistance

Among 23 tumors with acquired resistance from 20 patients, *EGFR* T790M secondary mutation was detected in 12 tumors (52%) from 11 patients (60%) (Table 2). *MET* amplification was detected in two tumors (9%) from two patients (10%). As HGF is a soluble cytokine, evaluation of HGF is not as simple as that for genetically conferred T790M secondary mutation and *MET* amplification, which can be designated as plus or minus. As described in the Materials and Methods section, we defined high-level HGF expression as H score $\geq$200 in this study. High-level HGF expression was detected in 14 tumors (61%) from 13 patients (60%). In these 14 tumors, HGF was predominantly expressed in cancer cells.

The high HGF expression was simultaneously detected in 6 of 12 tumors positive for T790M secondary mutation (50%) (Table 3, Figure 1). High-level HGF expression was also detected simultaneously in one of two tumors positive for *MET* amplification (50%). These results suggested possible interactions among these three resistance factors, consistent with previous reports.[16,17]

Expression of HGF, T790M Secondary Mutation, and MET Amplification in Tumors with Intrinsic Resistance (Nonresponders)

T790M secondary mutation was not detected in 45 tumors with intrinsic resistance from 44 patients (nonresponders), but *MET* amplification was detected in two tumors (4%) (Table 2). *EGFR* D761Y secondary mutation was detected in two tumors (4%) from one patient[24](Supplementary Table 1, http://links.lww.com/JTO/A197). In contrast, high-level HGF expression in cancer cells was detected in 11 tumors (24%) from 11 patients. In addition, HGF was detected at high levels in stromal cells in two tumors (4%) from two patients (data not shown). In total, high-level HGF expression was detected in 13 tumors with intrinsic resistance (29%). Notably, high-level HGF expression was simultaneously detected in one of two *MET* amplification-positive tumors (50%) (Table 2). These results suggested the involvement of HGF in intrinsic resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients.

Expression of HGF, T790M Secondary Mutation, and MET Amplification in Sensitive Tumors

Neither *EGFR* T790M secondary mutation nor *MET* amplification was detected in 29 sensitive tumors from 29 patients. High-level HGF expression was detected in two tumors (7%) (Supplementary Table 2, http://links.lww.com/JTO/A197). High levels of HGF were detected in stromal cells in one tumor (3%). In total, a high level of HGF expression was detected in three sensitive tumors (10%). Thus, although high HGF expression level was detected even in sensitive tumors, the incidence of high HGF expression was much lower in sensitive tumors than in those with acquired or intrinsic resistance. In addition, mean H score of HGF in tumors with acquired resistance was significantly higher than that in sensitive tumors ($p < 0.001$, Student's t test) (Figure 2). There was no significant difference in mean H score of HGF between tumors with intrinsic resistance (nonresponders) and sensitive tumors.

DISCUSSION

Our previous studies[14,25,26] documented HGF-mediated resistance to EGFR-TKIs in *EGFR* mutant lung cancer, which was also confirmed by other groups.[16,27] Here, we demonstrated that a high level of HGF expression was detected most frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. Our data indicated that although T790M secondary mutation and *MET* amplification are predominantly responsible for acquired resistance, HGF may be responsible not only for acquired resistance but also for intrinsic resistance to EGFR-TKIs.

The mechanism of intrinsic resistance to EGFR-TKIs is not well understood. To our knowledge, this is the first study with more than 40 clinical specimens indicating the incidence of resistance factors in intrinsic resistance to EGFR-TKIs in *EGFR* mutant lung cancer. Here, we found that a high level of HGF expression was most frequently (29%) detected in tumors with intrinsic resistance, compared with T790M secondary mutation (0%) and *MET* amplification (4%). It is noteworthy that although the high HGF expression level was detected in cancer cells in tumors with acquired resistance, HGF expression was detected in both cancer cells (10/12 tumors) and host stroma cells (2/12 tumors) in tumors with intrinsic resistance (nonresponders). HGF was reported to be produced by not only cancer cells but also stromal cells.[15] Our data clearly indicated that both cancer cells and stromal cells are sources of HGF, which induces intrinsic EGFR-TKI resistance in *EGFR* mutant lung cancer. As HGF-induced resistance could be reversed by anti-HGF antibody and the natural HGF inhibitor NK4,[25,27] highly produced HGF in

TABLE 3. Summary of Tumors with Acquired Resistance

ID	Gender	Histological Type	*EGFR* Mutation Status	Treatment	BOR	PFS	HGF	T790M	*MET* Amplification
KZ-1	M	Ad	Exon 19 del	Erlotinib	PR	254	60	–	+
KZ-2	F	Ad	L858R	Gefitinib	CR	1041	40	–	–
KZ-3	F	Ad	L858R	Gefitinib	PR	366	200	–	–
OK1—1	M	Ad	Exon 19 del	Gefitinib	PR	351	290	–	–
OK1—2							300	–	–
OK4—2	F	Ad	Exon 19 del	Gefitinib	PR	57	210	+	–
TS-1—3	F	Ad	L858R	Gefitinib	PR	180	90	–	–
TS-1—4							280	+	–
SG2	M	Ad	Exon 19 del	Gefitinib	PR	174	150	+	–
SG3	F	Ad	L858R	Gefitinib	SD	368	110	+	–
SG4	F	Ad	L858R	Gefitinib	PR	60	220	–	+
SG6	M	Ad	Exon 19 del	Gefitinib	PR	352	140	+	–
SG8	F	Ad	L858R	Gefitinib	SD	210	90	+	–
SG9	F	Ad	Exon 19 del	Gefitinib	SD	221	200	+	–
SG10	F	Ad	L858R	Gefitinib	CR	210	210	–	–
TB1—2	M	Ad	Exon 19 del	Gefitinib	PR	1770	230	+	–
TB2—2	F	AdSq	Exon 19 del	Gefitinib	PR	300	300	–	–
AC29—1	M	Ad	L858R	Gefitinib	PR	533	250	–	–
AC29—2							270	+	–
AC24	F	Ad	Exon 19 del	Gefitinib	PR	98	170	+	–
AC26	F	Ad	Exon 19 del	Gefitinib	SD	448	180	+	–
AC28	F	Ad	Exon 19 del	Gefitinib	PR	357	200	+	–
AC31	F	Ad	L858R	Gefitinib	PR	894	200	–	–

Ad, adeno; AdSq, adenosquamous; BOR, best overall response.

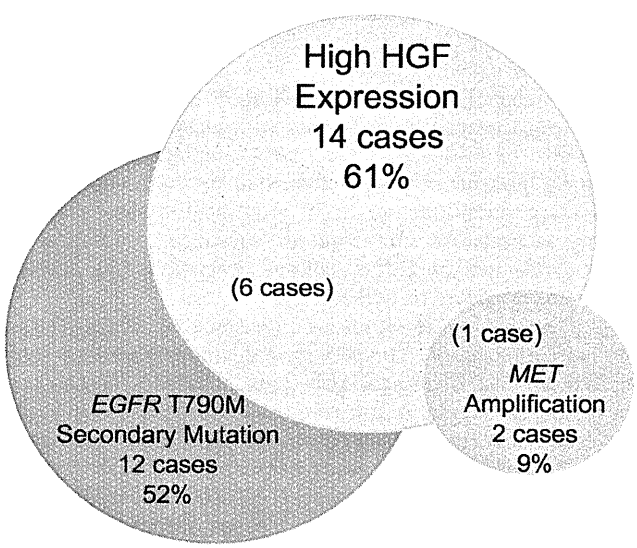

FIGURE 1. Incidences of high-level HGF expression, T790M secondary mutation, and *MET* amplification in 23 tumors with acquired resistance. Values in parentheses are the numbers of cases in which the tumors expressed two resistance factors simultaneously.

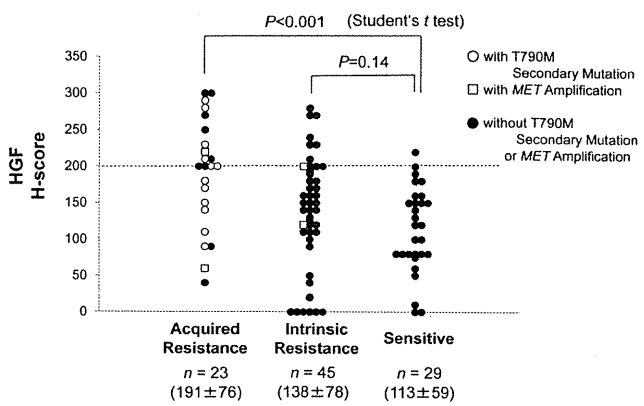

FIGURE 2. HGF expression score (*H* score) in EGFR-TKI-resistant tumors obtained from *EGFR* mutant lung cancer patients. Values in parentheses are mean ± SD of *H* score.

resistant tumors would be an ideal therapeutic target regardless of its origin.

It was of interest that a high level of HGF expression was detected in a small population of sensitive tumors. This was consistent with a previous report[16] indicating high-level HGF expression (*H* score ≥200) in several specimens from responders. Although the reason for the high level of HGF expression in tumors from responders is unclear at present, there are several possible explanations as follows. First, although HGF was expressed at high levels, natural inhibitors such as cleaved HGF and truncated MET, both of which inhibit binding of HGF to MET, may be generated in the tumors.[28,29] Second, negative regulators of MET tyrosine kinase activity such as protein kinase C may be activated and negate the effect of HGF on induction of EGFR-TKI resis-

tance in these tumors.[30] As the amounts of each clinical specimen were limited, we would like to perform further analyses in future studies should sufficient amounts of specimens become available.

Recent studies indicated that multiple resistance factors can be induced simultaneously in a single cancer. For example, Qi et al.[31] reported the simultaneous occurrence of *Met* mutation and activation of the EGFR pathway by ligand overexpression, similar to T790M mutation and HGF overexpression in EGFR mutant lung cancer, which caused resistance to Met-TKIs in gastric cancer. Katayama et al.[32] also reported that *ALK* gene amplification and gatekeeper mutation in *ALK* occurred simultaneously and conferred resistance to ALK inhibitors in EML4-ALK lung cancer. In this study, T790M secondary mutation and the high HGF expression level were simultaneously detected at high incidence (50%) in tumors with acquired resistance. Irreversible EGFR-TKIs were thought to have potential to control acquired resistance caused by T790M secondary mutation, but clinical responses were rarely observed in clinical trials.[33,34] We recently found that HGF induces resistance to not only reversible EGFR-TKIs but also irreversible EGFR-TKIs by activating the MET/PI3K/Akt pathway in *EGFR* mutant lung cancer cells with or without T790M secondary mutation.[26] Taken together, these observations suggest that HGF would be simultaneously expressed with T790M secondary mutation in tumors with acquired resistance and reduce the sensitivity to irreversible EGFR-TKIs in *EGFR* mutant lung cancer patients.

MET amplification has been detected in ~20% of tumors with acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer,[13,16,17] while the incidence reported in Japanese patients is rare.[14,18] Here, we detected *MET* amplification in two tumors (9%) with acquired resistance, suggesting that *MET* amplification can be detected in a significant proportion of tumors with acquired resistance even in Japanese patients. One case with high-level HGF expression and *MET* amplification (KZ-1) was treated with gefitinib and PFS was 254 days. The other case with low HGF and *MET* amplification (SG4) was treated with erlotinib and PFS was 60 days (Table 3). Although it is not possible to make definitive conclusions based on the data from only these two cases, the shorter PFS in the former case tentatively supports the observation that HGF accelerates expansion of preexisting clones with MET amplification.[16] Notably, simultaneous expression of these two factors was also detected in one tumor with intrinsic resistance (nonresponder). However, the mechanism by which HGF is induced in *EGFR* mutant lung cancer is still not well defined. Further examinations are warranted to elucidate the interaction between HGF expression and *MET* amplification in *EGFR* mutant lung cancer.

Among 68 resistant tumors, high-level HGF expression, T790M secondary mutation, and *MET* amplification were not detected in one tumor with acquired resistance and 31 tumors with intrinsic resistance, indicating the involvement of other mechanisms of resistance in these tumors. *EGFR* D761Y secondary mutation in exon 20 was detected in two tumors from the same patient.[24] *EGFR* D761Y mutation was originally identified in recurrent brain metastasis and was shown to induce intermediate-grade resistance to EGFR-TKIs.[35] In addition, rare secondary mutations (other than T790M and D761Y) or a preexisting resistance mutation in a minority of clones may also be involved in intrinsic resistance. Moreover, it was recently reported that a subpopulation of cancer cells that transiently exhibit a distinct phenotype characterized by engagement of IGF-1R activity, hypersensitivity to HDAC inhibition, and altered chromatin showed an intrinsic ability to tolerate exposure to EGFR-TKI.[36] Minor secondary mutations, a preexisting resistance mutation in a minority of clones, or chromatin-mediated drug resistance mechanisms may be involved in resistant tumors without high HGF expression, T790M secondary mutation, and *MET* amplification.

To overcome the HGF-induced resistance to EGFR-TKI in *EGFR* mutant lung cancer, double blockade of the EGFR pathway and HGF-MET pathway is therefore theoretically necessary.[14,16,27] To inhibit mutant EGFR with or without T790M secondary mutation, EGFR mutant-specific inhibitors were developed in addition to irreversible EGFR-TKIs.[37] To inhibit HGF-MET signaling, several inhibitors, including anti-HGF antibody, NK4 (natural antagonist of MET), and MET-TKIs, were developed.[16,25–27] Further studies are essential to determine optimal combined therapy with best efficacy and safety. In addition, a prospective study is required to determine whether immunohistochemical detection of HGF would be sufficiently reliable to identify patients with HGF-induced resistance to EGFR-TKIs. As levels of HGF in peripheral blood are correlated with clinical outcome to EGFR-TKIs in patients with non-small cell lung cancer,[38,39] such noninvasive methods may facilitate individual therapy for overcoming HGF-induced resistance to EGFR-TKIs in *EGFR* mutant lung cancer patients.

Recent studies indicated at least three important roles of HGF in EGFR-TKI resistance in *EGFR* mutant lung cancer. First, HGF induces resistance to reversible EGFR-TKIs, gefitinib, and erlotinib, by restoring MET/Gab1/PI3K/Akt pathways.[14,16] Second, HGF accelerates expansion of preexisting *MET*-amplified cancer cells and facilitates MET amplification-mediated resistance during EGFR-TKI treatment.[16] Third, after acquiring resistance to reversible EGFR-TKIs, HGF induces resistance of lung cancer cells with T790M secondary mutation to irreversible EGFR-TKIs.[24] Here, we detected high-level HGF expression frequently in tumors with intrinsic and acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer in Japanese patients. These findings indicate the value of HGF as a therapeutic target for EGFR-TKI-resistant *EGFR* mutant lung cancer. Therefore, combined therapy with EGFR-TKIs and HGF-MET inhibitors in patients with HGF-induced resistance may improve the clinical outcome of *EGFR* mutant lung cancer.

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S-1 Plus Cisplatin with Concurrent Radiotherapy for Locally Advanced Non-small Cell Lung Cancer

A Multi-Institutional Phase II Trial (West Japan Thoracic Oncology Group 3706)

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Purpose: To evaluate the combination chemotherapy using oral antimetabolite S-1 plus cisplatin (SP) with concurrent thoracic radiotherapy (RT) followed by the consolidation SP for locally advanced non-small cell lung cancer.

Patients and Methods: Patients with stage III non-small cell lung cancer, 20 to 74 years of age, and Eastern Cooperative Oncology Group performance status 0 to 1 were eligible. The concurrent phase consisted of full dose S-1 (orally at 40 mg/m^2/dose twice daily, on days 1–14) and cisplatin (60 mg/m^2 on day 1) repeated every 4 weeks for two cycles with RT delivered beginning on day 1 (60 Gy/30 fractions over 6 weeks). After SP-RT, patients received an additional two cycles of SP as the consolidation phase.

Results: Fifty-five patients were registered between November 2006 and December 2007. Of the 50 patients for efficacy analysis, the median age was 64 years; male/female 40/10; Eastern Cooperative Oncology Group performance status 0/1, 21/29; clinical stage IIIA/IIIB 18/32; and adenocarcinoma/others 20/30. There were 42 clinical responses including one complete response with an objective response rate of 84% (95% confidence interval [CI], 71–93%). The 1- and 2-year overall survival rates were 88% (95% CI, 75–94%) and 70% (95% CI, 55–81%), respectively. The median progression-free survival was 20 months. Of the 54 patients for safety analysis, common toxicities in the concurrent phase included grade 3/4 neutropenia (26%), thrombocytopenia (9%), and grade 3 esophagitis (9%) and febrile neutropenia (9%). In one patient, grade 3 pneumonitis was observed in the consolidation phase. There were two treatment-related deaths caused by infection in the concurrent phase.

Conclusions: SP-RT showed a promising efficacy against locally advanced NCSLC with acceptable toxicity.

Key Words: Concurrent chemoradiotherapy, Non-small cell lung cancer, Phase II trial, S-1, Cisplatin.

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The standard treatment modality in patients with unresectable stage III non-small cell lung cancer (NSCLC) is concurrent chemoradiotherapy.[1] Nevertheless, this combined treatment is associated with greater acute toxicity, including bone marrow[2] suppression, pneumonitis, and esophagitis,[2] compared with the sequential combination of chemotherapy and radiotherapy (RT). About a decade ago, we developed a concurrent chemoradiotherapy regimen using uracil-tegafur (UFT, Taiho Pharmaceutical Co., Ltd, Tokyo, Japan) plus cisplatin (UP) with concurrent thoracic RT (2 Gy per fraction, total 60 Gy) (UP-RT).[3] The response rate and median survival time of locally advanced unresectable stage III (IIIA 20%, IIIB 80%) patients treated with the UP-RT were 80% and 16.5 months, respectively, and these figures are similar to those reported in other concurrent chemoradiotherapy trials.[4,5] Nevertheless, the incidence of leukopenia and esophagitis of grade 3 or 4 was 16% and 3% of the patients, respectively,[3] and these figures are far lower than those of other trials.

S-1 (TS-1, Taiho Pharmaceutical Co., Ltd) is a second-generation oral anticancer agent based on uracil-tegafur, which has a dihydropyrimidine dehydrogenase (DPD) inhibitory fluoropyrimidine. S-1 comprises tegafur (a 5-FU Pro-

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Disclosure: The authors declare no conflicts of interest.

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drug), 5-chloro-2, 4-dihydroxypyridine (an inhibitor of DPD), and potassium oxonate (an inhibitor of phosphoribosyl transferase), in a molar ratio of 1:0.4:1 and has been shown to induce a comparable response to the other single agents for metastatic NSCLC.[6] Furthermore, combination chemotherapy using S-1 plus cisplatin (SP) for advanced NSCLC has been reported to show a response rate of 33 to 47% and a median survival time of 11 to 16 months.[7,8] Those data were better than the usual response rate of 29 to 38% and the median survival time of 8 to 13 months for the combination chemotherapeutic regimens using UP,[9,10] whereas the frequency of severe hematological and nonhematological adverse events induced by both UP and SP was lower than that of other platinum-based combination regimens such as carboplatin plus paclitaxel (CP), cisplatin plus docetaxel, and so on.[11–13] In addition, West Japan Oncology Group (WJOG) recently demonstrated that chemotherapy using S-1 plus carboplatin was noninferior in terms of overall survival (OS) compared with CP in advanced NSCLC.[14]

The above-mentioned observations indicated that it might be possible to use the same dose of SP as is used for metastatic advanced NSCLC for the treatment of locally advanced NSCLC with concurrent thoracic RT, similar to UP-RT. If this is possible, SP and concurrent thoracic RT (SP-RT) would be expected to provide several advantages over UP-RT. First, SP could have stronger antitumor activity for both locally advanced NSCLC and micrometastatic lesions than UP. Second, although both cisplatin and 5-FU have been reported to have a radiosensitizing effect,[15,16] the level of the latter in the blood by SP could not only be maintained at a higher level than by UP[17,18] but also 5-chloro-2, 4-dihydroxypyridine in S-1 has been recently reported to have a radiosensitizing effect as well as a strong DPD activity.[19,20] A single-institutional experience with SP-RT in 11 patients was reported showing that all the patients had a partial response, with acceptable hematological and nonhematological toxicities. On the basis of these findings, the WJOG (formally, West Japan Thoracic Oncology Group) conducted a multiinstitutional phase II trial to confirm the antitumor effects and safety of SP-RT.

PATIENTS AND METHODS

Eligibility Criteria

The eligibility requirements for enrollment in this phase II trial were cytologically or histologically confirmed, unresectable stage III NSCLC, for which radical dose RT could be prescribed. The staging was performed according to the 6th edition of tumor, node, metastasis (TNM). All patients were required to meet the following criteria: measurable disease; an Eastern Cooperative Oncology Group performance status of 0 or 1; a projected life expectancy of more than 3 months; a leukocyte count of $\geq$4000/μL; a platelet count of $\geq$100,000/μL; a blood gas oxygen level of $\geq$70 torr; a serum bilirubin level below 1.5 mg/dL; serum glutamic oxaloacetic transaminase/glutamic pyruvic transaminase levels of no more than 100 IU/ml; a creatinine level of $\leq$1.2 mg/dL; and a creatinine clearance level of $\geq$60 mL/min. Other eligibility criteria included no prior treatment and an age <75 years. All eligible patients underwent computed tomography (CT) scans of the thorax and upper abdomen and a radioisotope bone scan. Patients who had malignant pleural effusion, malignant pericardial effusion, a concomitant malignancy, or serious concomitant diseases were excluded from the study. Written informed consent was required from all patients, and the protocol was approved by the institutional ethics committee of each participating institute. All data were centrally monitored by the WJOG datacenter. This study is registered with the University Hospital Medical Information Network in Japan (number 000001370).

Treatment Schedule

Chemotherapy with SP

S-1 (40 mg/m^2/dose) in the form of 20 and 25 mg capsules containing 20 and 25 mg of tegafur, respectively, were taken orally twice a day after meals between days 1 and 14 as follows: in a patient with a body surface area (BSA) <1.25 m^2, 40 mg twice daily; for those with BSA 1.25 m^2, but <1.5 m^2, 50 mg twice daily; and BSA >1.5 m^2, 60 mg twice daily. Cisplatin (60 mg/m^2) was administered as a $\geq$120-minute infusion on day 1. The patients were also hydrated with 1000 to 2000 mL saline by infusion before cisplatin was administered. An antiemetic agent was administered at the discretion of each patient's physician.

The combination chemotherapy with SP was repeated twice, with a 4-week interval, concurrently with thoracic RT (SP-RT). At 2 to 4 weeks after the completion of the concurrent chemoradiotherapy, two further cycles of the same SP regimen were administered as a consolidation chemotherapy as shown in Figure 1.

A leukocyte count of 3000/μL or greater and the entry eligibility criteria regarding organ functions had to be satisfied for the patients to start the next cycle. If these criteria were satisfied 4 weeks after day 1 of each cycle of chemotherapy, the next cycle was administered. The doses of S-1 were adjusted according to the degree of hematological and nonhematological toxicity. The dose was reduced by one level (20 mg day) in patients whose BSA was 1.25 m^2 or more if there was evidence of grade 4 hematologic toxicity or grade 3 or more nonhematological toxicity during any cycle

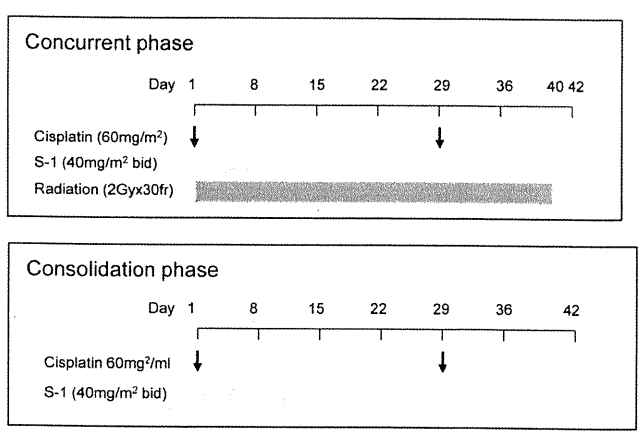

FIGURE 1. Treatment schedule.

of administration. If recovery from such toxicities was confirmed at a reduced dose, administration at the reduced dose was continued. If a patient with a BSA less than 1.25 m^2 experienced the above toxicities, then no further treatment with S-1 was performed. If a rest period of more than 4 weeks between two chemotherapy cycles of concurrent and consolidation phases was required or if the consolidation chemotherapy could not start within 6 weeks after SP-RT, then the SP treatment was discontinued.

Radiotherapy

All patients were treated with a linear accelerator photon beam of 6 MV or more from day 1. The primary tumor and involved nodal disease received 60 Gy in 2-Gy fractions over a period of 6 weeks. In this protocol, both 2- and 3-dimensional (D) treatment planning systems were allowed. The radiation doses were specified at the center of the target volume. The doses were calculated assuming tissue homogeneity without correcting for lung tissues for both 2- and 3-D treatment planning. Among the 54 patients assessable for toxicity, 2- and 3-D treatment planning were performed for 7 and 47 patients, respectively. The initial 40 Gy/20 fractions were delivered to clinical target volume 1 (CTV1), and the final 20 Gy/10 fractions were delivered to a reduced volume defined as clinical target volume 2 (CTV2). CTV1 included the primary tumor, ipsilateral hilum, and mediastinal nodal areas from the paratracheal (no. 2) to subcarinal lymph nodes (no. 7). For the primary tumors and the involved lymph nodes of 1 cm or more larger in the shortest diameter, a margin of at least 0.5 cm was added. The contralateral hilum was not included in CTV1. The supraclavicular areas were not treated routinely but were treated when the supraclavicular nodes were involved. CTV2 included only the primary tumor and the involved lymph nodes, with a margin of 0.5 to 1 cm. The spinal cord was excluded from the fields for CTV2 by appropriate methods, such as the oblique opposing method. The appropriate planning target volume margin and leaf margin were added for CTV1 and CTV2. When grade 4 hematologic toxicity, grade 3 to 4 esophagitis or dermatitis, pyrexia of $\geq$38°C, or a decrease in the partial pressure of arterial oxygen of 10 torr or more were compared with that before RT occurred, RT was interrupted. If a rest period of more than 2 weeks was required, then the patient was withdrawn from the study.

Evaluation of the Response and Toxicity

All registered patients, excluding those withdrawn from the study, received the following evaluations. Chest x-rays, complete blood cells, and blood chemistry studies were repeated once a week during the treatment period. Thoracic CT was performed every 1 or 2 months during the treatment period. After the treatment, a thoracic CT was obtained every 6 months, and other imaging examinations were obtained when recurrence was suspected. The response was evaluated in accordance with the RECIST version 1.0 guidelines.[21] In this study, the results of the response which an investigator determined were not used, and all responses were confirmed by the board members of the independent response review.

During the evaluation of both the initial staging and the antitumor effects, an extramural review was conducted. Only patients whose initial clinical stage was judged to be stage IIIA and IIIB and who were eligible for the study were analyzed for the response to treatment. The toxicity for all patients who received any treatment was assessed and graded by using the National Cancer Institute Common Terminology Criteria for Adverse Event version 3.

Statistical Analysis

The primary end point of this study was the objective tumor response rate. On the basis of the assumption that a response rate of higher than 80% would be expected from the combined modality treatment, while a rate below 60% would make a further investigation unnecessary, a sample size of 49 patients was required by the exact binomial test with a one-sided alpha error of 0.05 and a beta error of 0.1. Therefore, a total of 55 patients was the planned accrual size in view of possibly including ineligible patients. For determining the response rate, the exact binomial confidence interval (CI) was calculated. OS was defined as the time from registration until death from any cause. Progression-free survival (PFS) was defined as the time between registration and disease progression or death. The Kaplan-Meier method was used to estimate OS and PFS curves. All statistical analyses were done with SAS version 9.1.

RESULTS

Characteristics of Patients

Between November 2006 and December 2007, a total of 55 patients were enrolled from 18 institutes. One patient withdrew his consent and four patients were found to be ineligible by the extramural review (one malignant effusion, one carcinomatous lymphangitis, and 2 stage IV diseases). Therefore, the efficacy analyses were performed for the 50 remaining eligible patients. Safety analyses were performed for 54 patients who underwent SP-RT. Table 1 shows that 80% of the 50 eligible patients were male, with a mean age of 63 years (range, 40–74 years). Squamous cell carcinoma was the most common histological diagnosis, including 48% of the patients, and most patients had clinical stage IIIB disease (IIIA versus IIIB; 36% versus 64%). The most frequently classified TNM categories were T1-3N2 (36%), T1-3N3M0 (28%), and T4N0-1M0 (18%).

Adverse Events

The major adverse events (grade 3 and 4 toxicities) of SP-RT are listed in Table 2. Among the hematologic toxicities of the concurrent phase, grade 3 or higher leukopenia and neutropenia was observed in 17 patients (32%) and 14 patients (26%), respectively. Five patients (9%) developed grade 3 or higher thrombocytopenia. Among the nonhematologic toxicities, grade 3 and 4 febrile neutropenia was observed in four (7%) and one (2%) patient, respectively, whereas grade 3 esophagitis occurred in 4 patients (7%). Although no cases of severe pneumonitis occurred in the concurrent phase, two patients had a treatment-related death: one patient died of sepsis soon after the completion of the

TABLE 1. Patient Characteristics

No. of eligible patients	50
Age, yrs	
Mean (range)	63 (40–74)
Gender	
Male	40 (80%)
Female	10 (20%)
ECOG PS	
0	21 (42%)
1	29 (48%)
Smoking history	
Absent	2 (4%)
Present	48 (96%)
Histology	
Squamous cell carcinoma	24 (48%)
Adenocarcinoma	20 (40%)
Others	6 (12%)
cTNM	
Stage IIIA	18 (36%)
T1-3N2	18 (36%)
Stage IIIB	32 (64%)
T1-3N3M0	14 (28%)
T4N0-1M0	9 (18%)
T4N2M0	7 (14%)
T4N3M0	2 (4%)
Location of primary site	
Upper lobe	36 (72%)
Middle lobe	4 (8%)
Lower lobe	8 (16%)
Others	2 (4%)

ECOG, Eastern Cooperative Oncology Group; PS, performance status; TNM, tumor, node, metastasis.

TABLE 2. Hematological and Nonhematological Major Adverse Events

	Concurrent Chemoradiotherapy (n = 54)			Consolidation Chemotherapy (n = 39)		
	Grade			Grade		
Toxicities	3	4	Frequency of 3 or 4 (%)	3	4	Frequency of 3 or 4 (%)
Hematological						
Leukopenia	12	5	31.5	6	0	15.4
Neutropenia	10	4	25.9	4	0	10.3
Thrombocytopenia	1	4	9.3	4	0	10.3
Anemia	4	2	11.1	7	1	20.5
Nonhematological						
Febrile neutropenia	4	1	9.3	0	0	
Nausea	1	1	3.7	0	0	
Vomiting	2	0	3.7	0	0	
Anorexia	6	1	13.0	0	0	
Creatinine	0	0		0	0	
AST/ALT	1	1	3.7	0	0	
Diarrhea	2	0	3.7	0	0	
Stomatitis	1	0	1.9	0	0	
Pneumonitis	0	0		1	0	2.6
Esophagitis	4	0	7.4	1	0	2.6

AST, aspartate transaminase; ALT, alanine aminotransferase.

TABLE 3. Radiation Delivered (N = 50)

Radiation dose (Gy)	
Median (range)	60.0 (28–60)
Average	58.4
Reasons for interruption, N (%)	
Adverse events	7 (14.0)
Other	2 (4.0)
Rate of completion of treatment with 60 Gy, N (%)	47 (94.0)

TABLE 4. Chemotherapy Delivered (N = 50)

	N (%)
Concurrent chemotherapy	
Chemotherapy cycles	
1	50 (100)
2	46 (92.0)
Reasons for discontinuation	
Adverse event	2 (4.0)
Patient decision	2 (4.0)
Reasons for not proceeding to consolidation chemotherapy	
Adverse event	8 (16.0)[a]
Other	1 (2.0)
Consolidation chemotherapy	
Chemotherapy cycles	
1	37 (74.0)
2	31 (62.0)
Reasons for discontinuation	
Adverse event	5 (10.0)
Disease progression	1 (4.0)
Rate of completion of 4 cycles of treatment (95% CI)	62% (47.2–75.3)

[a] Two treatment-related deaths were included after completion of concurrent chemotherapy.
CI, confidence interval.

concurrent phase and the other patient died of pneumonia after the recovery from the bone marrow suppression because of that phase.

Thirty-nine (72%) of the 54 patients proceeded to consolidation chemotherapy. As shown in Table 2, the frequency of grade 3 or 4 in any major toxicity caused by consolidation chemotherapy was lower than that in concurrent chemoradiotherapy, except for anemia and pneumonitis. It was of note that no febrile neutropenia was observed.

Treatments Delivered to Eligible Patients

Tables 3 and 4 show RT and chemotherapy delivered to 50 eligible patients, respectively. Forty-six patients (92%) completed two cycles of SP concurrent with thoracic RT of 60 Gy. Two patients refused further protocol treatment after one cycle of chemotherapy because of adverse events. The other two patients did not meet the criteria to start the second cycle of SP because of prolonged neutropenia. Although 46 patients completed the concurrent phase of the SP-RT, seven patients could not proceed to the consolidation phase because of mainly prolonged hematological toxicity, and two patients

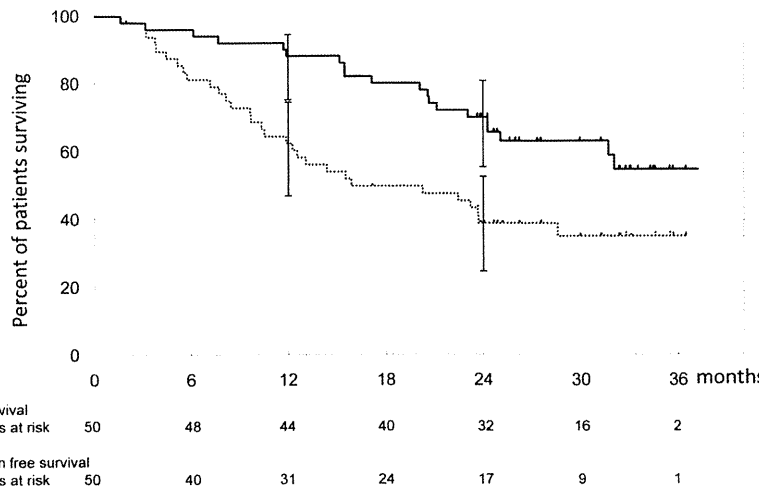

FIGURE 2. Overall survival (——) and progression-free survival (••••••). Each *tick* represents one patient who is alive with/without recurrence. The *bars* represent the 95% confidence intervals of the survival rate at 1 and 2 years after treatment.

were lost to treatment-related death. Of 37 patients, one and five patients received only one cycle of the consolidation chemotherapy because of disease progression and adverse events, respectively. Thus, a total of 31 (62%) of the 50 eligible patients received all four cycles of SP chemotherapy.

Response

Of the 50 patients eligible for efficacy analysis, 42 patients had responses (84%; 95% CI, 71–93%; $p < 0.0001$), including 1 patient with a complete response, and 2 patients with stable disease. Only one patient showed progressive disease. Five patients were unevaluable for a response. There were no differences in the response rate by histology (88% in squamous cell carcinoma versus 81% in others, $p = 0.704$ by the exact binomial test).

Survival

The overall median follow-up time for the 29 patients who were still alive as of January 2010 was 28 months (range, 24–37 months). As shown in Figure 2, the median PFS and OS was 20 months and not reached, respectively, and the OS rates at 1 and 2 years were 88% (95% CI, 75–94%) and 70% (95% CI, 55–81%), respectively.

Sites of First Failures

With respect to the sites of first failure among the 28 (56%) patients with disease progression of the 50 eligible patients, 19 (38%), 6 (21%), and 3 (6%) patients had distant metastases, intrathoracic local diseases, and both, respectively. Those nine occurred in the irradiated field. The frequently observed initial distant metastases were observed in bone in eight patients and in brain and lung in four each. Only four patients (8%) developed a brain metastasis alone as the initial failure site.

DISCUSSION

The purpose of concurrent chemoradiotherapy for NSCLC patients with stage III disease is to achieve local control, for which RT plays the main role, and also to eradicate occult distant metastases by chemotherapy. For the latter purpose, the development of regimens that can allow administration of the systemic (full) doses of chemotherapy during RT is necessary. Although the so-called "third generation" agents such as paclitaxel, vinorelbine, docetaxel, and gemcitabine have been evaluated in several concurrent studies in combination with platinum compounds, a lower dose of that agent plus the platinum compound has generally been used due to toxicities. Therefore, induction chemotherapy with sufficient systemic doses of the agents was considered a potentially effective addition to the concurrent chemoradiotherapy.[22] Nevertheless, a recent randomized trial (CALGB 39801) showed that two cycles of induction chemotherapy with full doses of CP did not provide a survival benefit over concurrent chemoradiotherapy alone, using weekly CP at lower doses.[23] Furthermore, the randomized phase III trial conducted by the Hoosier Oncology Group and U.S. Oncology Group demonstrated that the addition of consolidation chemotherapy using docetaxel after full-dose chemotherapy using cisplatin plus etoposide with concurrent RT (PE-RT) failed to achieve the primary end point of improved survival compared with PE-RT alone.[24] On the basis of these randomized trials, concurrent chemotherapy alone is recommended for the treatment of locally advanced-NSCLC. However, the optimal chemotherapy regimen remains to be determined.

In this study, SP-RT using systemic doses had the advantage of eradicating occult distant metastases. In addition, 5-FU has been reported to have a radiosensitizing effect in preclinical and clinical studies of various cancers, including NSCLC,[15,16] and S-1 is orally administered for 14 consecutive days in each course of chemotherapy, providing long-term potential radiosensitization. The antitumor effects of SP-RT might explain the high response rate of 82% and the prolonged median PFS of 20 months, as well as the median OS, which was not reached when follow-up time ranged from 24 to 37 months. Another SP-RT phase II trial with a similar schedule and dose, which was conducted during almost the same period as the present trial, also demonstrated a good overall response rate of 88%, median PFS of 12 months, and a median OS of 33 months, whereas the median follow-up

time was 25 months, ranging from 12 to 38 months.[25] Nevertheless, these data cannot be directly compared with our data. In this trial, the extraordinarily good results may not be only because of the chemotherapy regimen but also to the high frequency of the primary site being within the upper lobe. In completely resected NSCLC with N2 disease, the 5-year survival rate in patients with their primary site in the upper lobe is well known to be significantly better than that of patients with the primary tumor in the lower lobe.[26] In addition, the tumors in the upper lobe with upper mediastinal nodal metastases are easier to treat with RT than the tumors in the lower lobe in terms of the irradiation field.

Two additional cycles of the same chemotherapy after concurrent chemoradiotherapy were called consolidation chemotherapy in this trial. Although the original PE-RT regimen used two additional cycles of the same PE after PE (two cycles)-RT, the above-mentioned randomized trial did not use consolidation PE in both control and experimental groups.[24] Similarly, the original mitomycin, vindesine plus cisplatin (MVP)-RT regimen had the two additional cycles of the same MVP[5] after MVP (two cycles)-RT, whereas a recent randomized trial used MVP (two cycles)-RT alone as a control arm.[27] The median OS of PE (2 cycles)-RT and MVP (2 cycles)-RT was 23.2 and 23.7 months, respectively.[24,27] In addition, only 41% of the patients could complete four cycles of MVP in the MVP-RT group of a recent WJTOG phase III trial (WJTOG0105), which had a median OS of 20.5 months.[28] In this trial, 62% of the patients completed four cycles of SP despite a low frequency of severe toxicities, whereas the WJOG phase III trial showed that the safest regimen with concurrent RT was CP among MVP, CP, and carboplatin plus CPT-11, although the completion rate of two cycles in the consolidation chemotherapy of CP arm was only 50%.[28] These observations suggest that a phase III trial is necessary to clarify whether or not a total of four cycles of chemotherapy in this setting provides a better result than two cycles of chemoradiotherapy.

The irradiated dose of 60 Gy in 30 fractions with concurrent chemotherapy is currently used in the majority of institutes in Japan, whereas that of 66 Gy in 33 fractions in combination with chemotherapy in the United States seems to be the most common treatment regimen. Because PET/CT scan and 3-D planning were not used in all patients, it would therefore be interesting to elucidate whether or not the present survival of such patients can be prolonged by these techniques, including a total irradiated doses of 66 Gy.

Although the present treatment with SP-RT should be acceptably safe in terms of the frequency of grade 3 and 4 adverse events, the treatment-related death of two patients was observed. Therefore, it is necessary to keep in mind that there is no totally safe regimen for concurrent chemoradiotherapy. At present, the WJOG is conducting a randomized phase II trial comparing SP-RT to combination chemotherapy using cisplatin plus vinorelbine with concurrent RT.

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Clinicopathological findings of non-small-cell lung cancer with high serum progastrin-releasing peptide concentrations

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ABSTRACT

Although progastrin-releasing peptide (proGRP) is used as a serum tumor marker for small cell lung cancer (SCLC), high serum pro-GRP concentrations are observed in some non-small-cell lung cancers (NSCLCs). The characteristics of these NSCLCs are not well known. To determine the clinicopathological features of NSCLC in patients with elevated serum proGRP concentrations, serum proGRP values were assessed in 654 advanced lung cancer patients, and positive (>46 pg/mL) NSCLC specimens were subjected to cytological and histopathological reevaluation. Serum proGRP concentrations were positive in 34 of 421 NSCLC patients (8.1%) and 186 of 233 SCLC patients (80%). Histological subtypes of the 34 NSCLC patients at diagnosis were 20 adenocarcinomas, 5 squamous cell carcinomas, 4 large cell carcinomas, and 5 large cell neuroendocrine carcinomas. Six of 27 cytology specimens contained characteristic neuroendocrine morphology. Immunohistochemical analysis showed that 11 of 17 tumors were positive for neuroendocrine markers (64.7%). Twenty of 34 serum proGRP-positive NSCLC patients received platinum-based chemotherapy, and the response rate was 55.0%. These results suggest that serum proGRP-positive NSCLCs may have neuroendocrine differentiation. In addition, serum proGRP-positive NSCLCs may have clinical characteristics that are different from other NSCLCs.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide. In 2005, the number of deaths due to lung cancer in Japan exceeded 60,000 [1]. Conventionally, lung cancer is classified into small cell lung cancer (SCLC) and non-small-cell carcinoma (NSCLC). Because SCLC has neuroendocrine features, it has a poorer prognosis and shows greater sensitivity to chemotherapy than NSCLC. Although NSCLC is subclassified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, some NSCLCs have neuroendocrine differentiation. In 1999, the World Health Organization categorized large cell neuroendocrine carcinoma (LCNEC) as a variant of large cell carcinoma [2]. LCNEC has been reported to have a poor prognosis, even for early-stage disease [3,4]. Different types of NSCLCs differ in their clinical behavior according to the presence or absence of neuroendocrine differentiation. Neuroendocrine differentiation in a tumor is generally determined by immunohistochemistry and/or electron microscopy, which reveal the characteristic neuroendocrine morphology [2,5]. However, it is difficult to obtain sufficient tissue by biopsy, and limited tumor tissue sampling may make it difficult to diagnose neuroendocrine differentiation in NSCLC. Therefore, the development of a sensitive serum marker for the detection of neuroendocrine differentiation is greatly desired to facilitate the diagnosis of NSCLCs and neuroendocrine tumors.

Progastrin-releasing peptide (proGRP) is a signal peptide that is produced by small cell lung cancer cells (SCLC). Serum proGRP is considered to be a sensitive tumor marker for SCLC. The sensitivity and specificity of serum proGRP as a tumor marker for SCLC is 60–70% and 96%, respectively [6]. Elevated serum pro-GRP concentrations have been observed in some NSCLC patients, especially LCNEC patients [6,7], suggesting that serum proGRP is a potentially good marker not only for SCLC but also for NSCLC with neuroendocrine features. However, the clinical and pathological characteristics of NSCLCs with elevated serum proGRP concentrations have not been well studied. In the present study, serum proGRP levels were measured in 654 lung cancer patients and the clinical characteristics of serum proGRP-positive NSCLC patients

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were analyzed; the histopathology of the surgical or biopsy specimens of the positive patients were also evaluated.

2. Patients and methods

Serum proGRP concentrations were measured in 654 patients who were diagnosed with lung cancer by histology or cytology at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research between April 1998 and April 2006.

An enzyme-linked immunosorbent assay (ELISA) kit (serumlabo ProGRP; Fujirebio Diagnostics Inc., Tokyo, Japan) was used to determine serum proGRP concentrations, and samples were considered positive when their values exceeded 46 pg/mL [8].

The clinical characteristics of serum proGRP-positive NSCLC patients were retrospectively analyzed, including age at diagnosis, gender, smoking history, and TNM stage. Response to platinum-based chemotherapy in serum proGRP-positive NSCLC patients was determined according to RECIST criteria (without confirmation).

In addition, the cytological and histological findings of the surgical or biopsy specimens of these patients were reevaluated. Immunohistochemical (IHC) staining was used to evaluate neuroendocrine differentiation in the tumors. Formalin-fixed paraffin-embedded sections were stained for a panel of epithelial markers, including thyroid transcription factor-1 (TTF-1; Dako EnVision+, Saitama, Japan) and carcinoembryonic antigen (CEA; Nichirei, Tokyo, Japan), and neuroendocrine markers, including chromogranin A (CGA) (Dako EnVision+, Saitama, Japan), synaptophysin (Dako EnVision+, Saitama, Japan), CD56 (neural cell adhesion molecule [NCAM]) (Clone 1B6; Novocastra, and proGRP (Advanced Life Science Institute Inc., Saitama, Japan). IHC staining was performed according to standard protocols with EnVision kits (Dako EnVision+, Saitama, Japan). IHC results were grouped into 3 categories – strongly positive, weakly positive, or negative – by well-trained pathologists (WH and NM).

Statistical calculations were performed using StatView version 5.0 for Windows XP (SAS Institute, Cary, NC). Associations between categorical variables and serum proGRP concentrations were evaluated using Student's *t* test. Survival was measured from the start of chemotherapy to the last follow-up evaluation or death, and survival rates were estimated using the Kaplan–Meier method.

3. Results

3.1. Patient characteristics

Of a total of 654 patients, 421 were diagnosed with NSCLC and 233 with SCLC. Serum proGRP samples were positive in 220 of 654 patients, of which 34 (8.1%) had NSCLC and 186 (80%) had SCLC.

The clinical characteristics of serum proGRP-positive and negative NSCLC patients are shown in Table 1. There were no significant differences in the clinical characteristics between the serum proGRP-positive and -negative NSCLC patients.

In serum ProGRP-positive NSCLC patients, the median age of these patients was 67 years (range, 49–77). There were 22 males and 12 females, and 65% of the patients were heavy smokers (smoking index > 400). Most of the patients (94%) had advanced NSCLC. Serum creatinine concentrations were less than 1.6 mg/dL in all 34 serum proGRP-positive NSCLC patients.

The histological subtypes of the 34 serum proGRP-positive NSCLCs at diagnosis were as follows: 20 adenocarcinomas, 5 squamous cell carcinomas, 4 large cell carcinomas, and 5 LCNECs. The rates of positive serum proGRP in each histological subtype were as follows: 7.7% in 260 adenocarcinomas, 5.9% in 85 squamous cell carcinomas, 9.3% in 43 large cell carcinomas, and 44.4% in 11 LCNECs.

Table 1
Clinical characteristics of NSCLC patients.

Characteristics	ProGRP-positive NSCLC patients	ProGRP-negative NSCLC patients
Total no. of patients	34	387
Age, years		
Median (range)	67 (49–77)	62 (29–87)
Sex		
Male/female	22/12	261/126
Smoking index		
Mean (range)	807.5 (0–1400)	661 (0–3000)
Never/≤400/>400	10/2/22	121/29/237
Histological subtype at diagnosis		
Adenocarcinoma	20	240
Squamous cell carcinoma	5	80
Large cell carcinoma	4	48
LCNEC	5	6
Adenosquamous	0	2
Other	0	11
Stage		
I/II	2	56
IIIA	7	63
IIIB	6	106
IV	16	144
Recurrent	3	18

LCNEC: large cell neuroendocrine carcinoma, ProGRP: progastrin-releasing peptide, NSCLC: non-small-cell lung cancer.

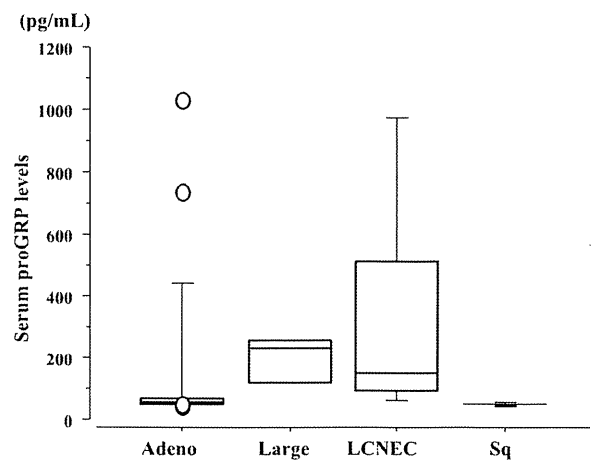

Fig. 1. Serum progastrin-releasing peptide (proGRP) concentrations of 34 non-small-cell lung cancer patients with elevated proGRP. Adeno: adenocarcinoma, Large: large cell carcinoma, LCNEC: large cell neuroendocrine carcinoma, Sq: squamous cell carcinoma.

The median serum proGRP concentration of the 34 NSCLC patients was 60.7 pg/mL and the range was 46.0–973.0 pg/mL. The serum proGRP concentrations in these 34 NSCLC patients were significantly lower than the serum concentrations in proGRP-positive SCLC patients (median, 469 pg/mL; range, 47.1–344,000 pg/mL) ($P < 0.05$).

Fig. 1 shows the serum proGRP concentrations for each histological subtype of serum proGRP-positive NSCLC. The mean serum proGRP concentration in LCNECs was 147 pg/mL and the range was 78.6–973 pg/mL. These concentrations are relatively high compared to other NSCLCs. On the other hand, serum proGRP concentrations were relatively low, even in serum proGRP-positive squamous cell carcinoma patients (median, 47.4 pg/mL; range, 46–56.7 pg/mL).