Table 3. *EGFR* mutations among patient subgroups

	n	*EGFR* mutations				*P*
		DEL	L858R	Total	%	
Total	207	49	36	85	41	
Sex						
Women	89	31	17	48	54	0.001
Men	118	18	19	37	31	
Smoking history						
Never smokers	93	30	19	49	53	0.002*
Former smokers	38	12	10	22	58	
Current smokers	76	7	7	14	18	
Histology						
Adenocarcinoma	189	48	35	83	44	0.007
Others	18	1†	1‡	2	11	

*Comparison between never smokers and others.
† Pleomorphic carcinoma.
‡ Adenosquamous carcinoma.

Clinical validation of HRMA. Direct sequencing with and without laser capture microdissection had been done in 66 patients with recurrent NSCLC after surgery in the previous study (6). In these patients, HRMA was done using both formalin-fixed and methanol-fixed surgical specimens without laser capture microdissection, and the results were compared with the results of direct sequencing with laser capture microdissection, which we considered to be the gold standard method.

Radiologic evaluation. One board-certified radiologist (U.T.) who was unaware of the patients' mutational statuses reviewed the baseline, the first follow-up, and confirmatory imaging studies and classified the tumor responses into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) using standard bidimensional measurements (11). In patients without measurable lesions, significant clinical benefit and disease progression were defined as clinical PR and clinical PD, respectively. Patients who died before the follow-up imaging studies were classified as PD. SD was subdivided into minor response (MR), long SD, and short SD. MR was defined as a ≥25% decrease in the sum of the products of the perpendicular diameters of all measurable lesions, and long SD meant that SD lasted for >6 months. Responders were defined as patients with CR, PR, or clinical PR.

Statistical analysis. The associations among *EGFR* mutations, patient characteristics, and tumor responses to gefitinib were assessed using a χ^2 test. The differences in time-to-progression and overall survival according to the patient subgroups were compared using Kaplan-Meier curves and log-rank tests. The starting point of the time-to-progression and overall survival was the first administration of gefitinib. Multivariate analyses using logistic regression models and Cox proportional hazard models were done to assess the association between the clinical outcomes and the following factors: age (<70 versus ≥70 years), sex, smoking history (never smokers versus others), histology (adenocarcinoma versus others), performance status (0/1 versus 2/3), stage (recurrence after surgery versus III/IV), prior chemotherapy (yes versus no), and the mutational status of *EGFR* (mutant versus WT). All analyses were done using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS, Inc.).

Results

Patient characteristics. The patient characteristics are listed in Table 1. All the patients were East Asians: 210 Japanese, 1 Korean, and 1 Chinese. The median follow-up time for the survivors was 29.7 months (range, 10.7-49.8 months).

Clinical validation of HRMA. The clinical validation of the HRMA results using various samples is shown in Table 2. The sensitivity of HRMA using DNA extracted from formalin-fixed tissues was 92%, significantly higher than that of direct sequencing without laser capture microdissection but lower than that of HRMA using methanol-fixed tissues. The specificity and positive predictive values were 100% in all the analyses.

Mutational analysis. HRMA was completed in 207 patients. Five patients could not be successfully analyzed because of incomplete PCR. Of the 207 patients, 130 were analyzed using tissue samples (96 samples were obtained by thoracotomy, 17 by mediastinoscopic lymph node biopsy, 9 by thoracoscopic lung or pleural biopsy, 5 by resection or biopsy of distant metastases, and 3 by transbronchial lung biopsy), and 117 were analyzed using cytology samples (43 samples were obtained by bronchial brushing or washing, 40 from pleural effusion, 9 by transbronchial needle aspiration, 8 from pericardial effusion, 7 by needle aspiration of superficial lymph nodes, 6 by percutaneous needle aspiration of lung tumors, and 4 from sputum). In 40 patients who were analyzed using both tissue and cytology samples, 4 had inconsistent results; mutations were detected only in tissue samples and not in cytology samples (3 patients) or vice versa (1 patient). These four patients were judged to have mutations because false-negative results were more common than false-positive results in the validation of HRMA. Consequently, DEL and L858R mutations were detected in 49 (24%) and 36 (17%) patients, respectively, and these mutations were mutually exclusive. The other 122 (59%) patients were classified as having WT *EGFR* in this study, although some of them may have had minor mutations. As

Table 4. *EGFR* mutations and response to gefitinib

	Responders		SD			PD	Response rate (%)	*P*
	CR	PR	MR	Long SD	Short SD			
WT	0	10	2	4	17	89	10/122 (8)	$<10^{-23}$
Mutant	2	64*	6	4	1	8†	66/85 (78)	
DEL	0	42	2	2	1	2	42/49 (86)	0.037
L858R	2	22	4	2	0	6	24/36 (67)	
Total	2	74	8	8	18	97	76/207 (37)	

*Including four clinical responders without measurable lesions.
† Including a patient who had no measurable lesions at baseline.

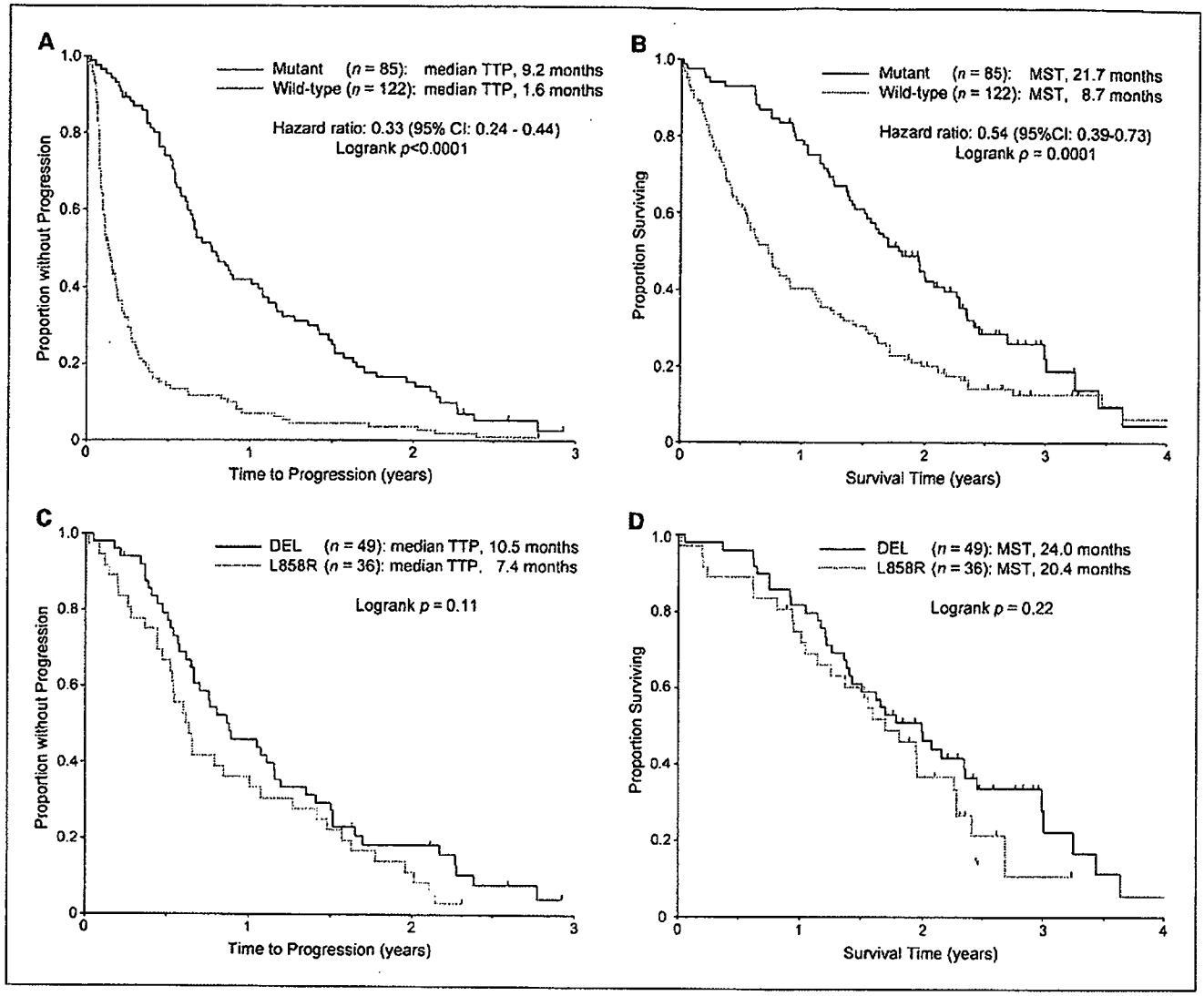

Fig. 1. Kaplan-Meier plot of time-to-progression (*A*) and overall survival (*B*) for patients with or without *EGFR* mutations. Kaplan-Meier plot of time-to-progression (*C*) and overall survival (*D*) for patients with DEL or L858R mutations. TTP, time-to-progression; MST, median survival time.

shown in Table 3, *EGFR* mutations were detected more frequently in women, never smokers, and patients with adenocarcinoma. Patient characteristics were not significantly different between patients with DEL mutations and those with an L858R mutation.

EGFR mutations and clinical outcomes. The association of the mutational status of *EGFR* and the response to gefitinib is shown in Table 4. The response rate was significantly higher in patients with *EGFR* mutations than in those with WT *EGFR* (78% versus 8%; $P < 10^{-23}$). Among patients with *EGFR* mutations, those with DEL mutations had a higher response rate than those with an L858R mutation (86% versus 67%; $P = 0.037$). Tumor responses were classified as SD in 11 patients with *EGFR* mutations and in 23 patients with WT *EGFR*. Among the patients with SD, a MR and/or a long SD (>6 months) were observed more frequently (91% versus 26%; $P = 0.0004$) and the time-to-progression was significantly longer (median, 6.9 versus 4.4 months; $P = 0.019$) in the patients with *EGFR* mutations than in the patients with WT *EGFR*.

As shown in Fig. 1, the time-to-progression (median, 9.2 versus 1.6 months; $P < 0.0001$) and overall survival (median, 21.7 versus 8.7 months; $P = 0.0001$) were significantly longer in patients with *EGFR* mutations than in those with WT *EGFR*. Patients with DEL mutations tended to have a longer time-to-progression (median, 10.5 versus 7.4 months; $P = 0.11$) and overall survival (median, 24.0 versus 20.4 months; $P = 0.22$) than those with an L858R mutation, although the difference did not reach statistical significance.

Clinical outcomes among subgroups of patients are shown in Table 5. In the univariate analysis, sex, smoking history, and histology were significant predictive factors for gefitinib sensitivity.

In the multivariate analyses, the mutational status of *EGFR* was an independent predictive factor of response [odds ratio, 38.9; 95% confidence interval (95% CI), 15.7-96.5; $P < 0.001$], time-to-progression (hazard ratio, 0.33; 95% CI, 0.24-0.45; $P < 0.001$), and overall survival (hazard ratio, 0.48; 95% CI, 0.34-0.67; $P < 0.001$). A poor performance status (2/3) was an

independent predictor of a shorter time-to-progression (hazard ratio, 1.80; 95% CI, 1.19-2.72; $P = 0.006$) and overall survival (hazard ratio, 3.97; 95% CI, 2.56-6.16; $P < 0.001$), and a history of prior chemotherapy was another independent predictor of a shorter overall survival (hazard ratio, 1.59; 95% CI, 1.14-2.23; $P = 0.006$). However, other clinical characteristics, including sex, smoking history, and histology, were not independent predictive factors for any clinical outcomes.

Discussion

In the current study, we showed the practicality of our new HRMA method for detecting two major *EGFR* mutations, DEL and L858R. The sensitivity and specificity of the analysis were 92% and 100%, respectively, when archived formalin-fixed, paraffin-embedded tissues were used without laser capture microdissection. Given the similar results that were obtained when Papanicolaou-stained cytologic slides were used (10), DEL and L858R mutations can likely be detected from such archived samples with about a 90% sensitivity and 100% specificity. Because the mutations were detected by HRMA even when only a small proportion (0.1% or 10%) of mutant cells existed (10), laser capture microdissection or other enrichment procedures are not needed in most cases. This is a major advantage of HRMA over direct sequencing because direct sequencing requires laser capture microdissection for accurate evaluation (6). However, there remained some risk of indeterminate or false-negative results because the DNA might have degenerated during sampling or the preservation of the archived samples. In fact, an analysis using methanol-fixed tissues, which are known to preserve DNA better than formalin-fixed tissues (12), was stable with no indeterminate and fewer false-negative results. Thus, an even higher sensitivity can be expected when fresh tumor samples are used. In any event, HRMA was successfully used to identify *EGFR* mutations and, more importantly, predict the clinical outcomes of gefitinib-treated patients with a high sensitivity and specificity.

Although the detection of *EGFR* mutations can provide patients with NSCLC and their physicians with critical information for optimal decision making, such tests are not common in clinical settings mainly because of the difficulty and impracticality of direct sequencing. Recently, highly sensitive nonsequencing methods to detect *EGFR* mutations in small tumor samples contaminated with normal cells have been reported (10, 13-21). Among them, HRMA has the advantages of being able to identify the mutations with less labor, time, and expense; PCR and the melting analysis can be done in the same capillary tube within a few hours, and the running cost is only about 1 U.S. dollar per sample. HRMA is expected to be one of the most practical methods for detecting *EGFR* mutations in clinical settings.

We analyzed consecutive gefitinib-treated patients in a single institution on a larger scale than any other previous report. The mutational analysis by HRMA was successful in 207 patients and confirmed strong and independent associations between the two major *EGFR* mutations and clinical outcomes. Clinical predictors, such as sex, smoking history, and histology, added little predictive information to that provided by the mutational analysis. We believe that the mutational status of *EGFR* is the most important predictor of clinical outcomes in gefitinib-treated patients.

Among the patients without the two major mutations, 8% were responders. This result may be due to false-negative HRMA results, other *EGFR* mutations, or other determinants of gefitinib sensitivity. As for other *EGFR* mutations, the direct sequencing of exons 18 to 24 was done in four responders without DEL or L858R mutations, and one of them had G719C and S768I mutations. Although missense mutations at codon 719 of *EGFR* (G719C, G719S, or G719A) may be associated with gefitinib sensitivity, the predictive significance of these mutations is unclear because the number of reported patients is small (6). At present, we consider the accurate detection of the two major *EGFR* mutations to be sufficient for optimal decision making.

Recently, the *EGFR* copy number was reported to be another predictor of gefitinib sensitivity (6, 22, 23), and Cappuzzo et al. (22) suggested that this factor was a stronger predictor of overall survival than *EGFR* mutations. Our previous study also showed that the *EGFR* copy number evaluated by quantitative

Table 5. Clinical outcomes among subgroups of patients

	n	Response rate (%)	*P*	Median TTP (mo)	*P*	MST (mo)	*P*
Total	207	37		3.7		14.5	
Sex							
Women	89	51	<0.001	5.6	0.17	18.3	0.15
Men	118	26		2.3		9.6	
Smoking history							
Never smokers	93	51	<0.001*	6.2	0.073*	16.9	0.22*
Former smokers	38	47		5.2		14.5	
Current smokers	76	14		2.2		9.1	
Histology							
Adenocarcinoma	189	40	0.004	4.3	0.060	15.1	0.10
Others	18	6		1.6		4.9	
EGFR mutations							
DEL/L858R	85	78	<0.001	9.2	<0.001	21.7	<0.001
WT	122	8		1.6		8.7	

Abbreviations: TTP, time-to-progression; MST, median survival time.
*Comparison between never smokers and others.

PCR was associated with response; however, an increased *EGFR* copy number was concentrated in patients with *EGFR* mutations and was not an independent predictor of response and overall survival (6). In the current study, we showed that *EGFR* mutations were associated with better outcomes even among patients with SD. The interpretation of this result is difficult because a long SD might be caused by intrinsic characteristics independent of treatment; however, this result suggested that *EGFR* mutations predicted not only "super responders" but also "non-super responders" who gained a clinical benefit. Contrary to these findings, Cappuzzo et al. (22) showed that *EGFR* mutations predicted only responders and were not associated with overall survival, whereas *EGFR* copy number was associated with both response and SD and was an independent predictor of overall survival. Although the reason of these discrepancies is unclear, we consider that if *EGFR* mutations are accurately identified, *EGFR* copy number adds little information for patient selection, at least in Japanese patients.

About the outcomes of patients with DEL or L858R mutations, our larger scale study produced results similar to those of some previous studies, which indicated that DEL mutations were associated with better outcomes after EGFR tyrosine kinase inhibitor treatment than an L858R mutation (24–27). Further investigations are needed to clarify the difference in the biological characteristics of the two mutations. However, in the current study, the difference was small and even patients with an L858R mutation had favorable outcomes: the response rate was 67%, the median time-to-progression was 7.4 months, and the median survival time was 20.4 months. We now think that both DEL and L858R mutations should be treated equally in clinical decision-making.

In conclusion, the detection of DEL and L858R mutations using HRMA is accurate and practical. Using HRMA, we confirmed a strong association between the two major *EGFR* mutations and clinical outcomes in patients with advanced NSCLC treated with gefitinib.

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Epidermal growth factor receptor mutation status and clinicopathological features of combined small cell carcinoma with adenocarcinoma of the lung

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In lung cancer, somatic mutations of epidermal growth factor receptor (*EGFR*) are concentrated in exons 18–21, especially in adenocarcinoma (Ad), but these mutations have rarely been reported in small cell lung carcinoma (SCLC). Combined SCLC is rare, and the *EGFR* mutation status and its relationship to the clinicopathological features of this tumor type have not yet been elucidated. We retrospectively studied six patients with combined SCLC with Ad components among 64 consecutive patients who underwent resection of SCLC. The clinicopathological features of each patient were reviewed, especially for the distribution pattern of the Ad component and lymph node metastases. *EGFR* mutations were screened by high-resolution melting analysis in each case, and were confirmed by sequencing of each mutation in the microdissected SCLC or Ad components. Regarding *EGFR*, no specific mutation was detected in five of the six patients, whereas one female patient who had never smoked had a missense mutation. In this case, both the SCLC and Ad components shared the same mutation in exon 21 (L858R). We identified a patient with combined SCLC with Ad sharing an identical *EGFR* mutation in both the SCLC and Ad components. In addition to the clinicopathological characteristics of this rare histological type of lung cancer, these findings provide useful information for better understanding the biology, natural history and clinical management of SCLC. (*Cancer Sci* 2007; 98: 1714–1719)

Small cell lung carcinoma (SCLC) accounts for 15–20% of all lung cancers worldwide.[1] SCLC is known to be more sensitive than non-SCLC to chemotherapy, but shows a more aggressive clinical course. The median survival time without treatment is 2–4 months.[2,3] Approximately 20% of patients with limited SCLC achieve a cure, but most patients with SCLC will relapse, and relapsed or refractory SCLC has a uniformly poor prognosis with a 5-year survival rate of less than 5%.[4]

According to the 2004 World Health Organization (WHO)/International Association for the Study of Lung Cancer (IASLC) classification of lung and pleural tumors,[5] 'combined SCLC' is defined as SCLC combined with an additional component that consists of any of the histological types of non-SCLC, usually adenocarcinoma (Ad), squamous cell carcinoma (Sq) or large cell carcinoma. Combined SCLC is rare, and has been reported to account for less than 1–3.2% of all SCLC.[6,7] However, a high proportion (12–26%) of SCLC patients who undergo surgical resection show combination with non-SCLC.[8–12]

In a clinical setting, the distinction of SCLC from non-SCLC is critical because of major differences in patient management and prognosis. Recently, molecular targeted therapy has been developed using agents such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which exerts antitumor activity in patients with advanced non-SCLC (especially Ad) with *EGFR* mutations. High expression of EGFR has been reported in various epithelial malignant tumors, including lung cancer,[13,14] and somatic mutations in the kinase domain of *EGFR* are suggested to be strongly correlated with sensitivity to EGFR tyrosine kinase inhibitor.[15,16] These mutations are concentrated in exons 18–21 of *EGFR*, and approximately 90% of *EGFR*-mutant patients with lung Ad have mutations in two hot spots: in-frame deletion at codons 747–749 (DEL) in exon 19, and a missense mutation at codon 858 (L858R) in exon 21.[17,18] Although these mutations have rarely been reported in SCLC, two recent studies have demonstrated *EGFR* mutation in SCLC.[19,20]

In the present study, we retrospectively investigated six resected cases of combined SCLC with an Ad component to elucidate the clinicopathological features of this rare tumor, especially the ratio of each tumor component, the distribution patterns of the Ad component, and the status of lymph node metastasis. The *EGFR* mutation status in surgically resected specimens was also analyzed for each histological type in the same tumor.

Materials and Methods

Patients and histological diagnosis. A search of our surgical pathology files covering the period January 1982 to December 2004 yielded 64 consecutive patients with SCLC who had undergone surgical resection at the National Cancer Center Hospital, Tokyo, Japan. For the purposes of the present study, we identified six patients with combined SCLC with an Ad component. The research protocol was approved by the Institutional Review Board.

The surgically resected specimens were fixed in 10% formalin. All sections containing both tumor tissues and surrounding lung tissues were embedded in paraffin. Additional consecutive 5 μm-thick sections were cut from the tissue block and stained with hematoxylin and eosin. All histological diagnoses were reviewed by certificated pathologists (K. T., A. M. M. and Y. M.) based on the most recent WHO/IASLC classification of lung and pleural tumors.[5] Both clinical and pathological staging data for each patient have been reported according to the International Staging System for Lung Cancer.[21] Patient survival was calculated as the time between operation and death.

Immunohistochemistry and evaluation. For phenotypic analysis, paraffin section immunohistochemistry was carried out using the primary antibodies listed in Table 1, followed by subsequent labeling with the Envision+ horseradish peroxidase (HRP) system (DAKO, Carpinteria, CA, USA). For heat-induced epitope retrieval, sections stained for p63 were treated with 1.0 mmol/L

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Table 1. Results of immunohistochemistry

Patient no.	SCLC component (%)	Immunoreaction					Non-SCLC component (%)	Immunoreaction					No. tumor embolism cells per slice[1] (%)		
		CgA	SYN	NCAM	TTF-1	p63		CgA	SYN	NCAM	TTF-1	p63	SCLC	Ad	Sq
1	95	2+	3+	3+	3+	0	Ad, 5	1+	1+	1+	3+	0	30 (97)	1 (3)	–
2	80	3+	3+	3+	3+	0	Ad, 10	0	1+	1+	2+	1+	21 (84)	3 (12)	1 (4)
							Sq, 10	0	0	0	0	3+			
3	70	1+	3+	3+	3+	0	Ad, 30	0	1+	0	3+	0	38 (93)	3 (7)	–
4	55	2+	3+	3+	3+	0	Ad, 45	0	0	1+	1+	0	24 (92)	2 (8)	–
5	35	3+	3+	3+	3+	0	Ad, 60	1+	1+	1+	3+	1+	17 (100)	0 (0)	0 (0)
							Sq, 5	0	1+	0	0	2+			
6	5	Not done					Ad, 95	Not done					Not done		

CgA, chromogranin-A; NCAM, neural cell adhesion molecule; SCLC, small cell lung carcinoma; SYN, synaptophysin; TTF-1, thyroid transcription factor-1. Semiquantitative assessments of the percentage of positive tumor cells (0 = none, 1+ = 1–33%, 2+ = 34–66%, 3+ = 67–100%) were made. [1]We counted the number of lymph vessels with tumor embolisms confirmed by staining for D2-40 for a representative slide.

Table 2. Clinical characteristic of the patients with combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad)

Patient no.	Age/Sex	ECOG PS	Smoking status	Smoking index	Tumor location	Size (mm)	Stage (cTNM)	Preoperative diagnosis	Surgical procedure
1	74/Male	0	Current	2160	Peripheral	31	IIb (210)	Unknown	RLL[1]
2	66/Male	0	Ever	900	Peripheral	38	IIb (210)	Unknown	RM/LL[2]
3	62/Female	0	Never	0	Peripheral	31	Ib (200)	SCLC	LUL
4	77/Male	1	Current	570	Peripheral	15	Ia (100)	Unknown	Left pneumonectomy
5	75/Male	0	Ever	1000	Peripheral	30	Ia (100)	Non-SCLC	RUL
6	76/Male	0	Current	1120	Peripheral	28	Ia (100)	Ad	RUL

Smoking index: (number of cigarettes smoked per day) × years. Adjuvant chemotherapy: [1]cyclosphosphamide + doxorubicin + vincristine × 1 cycle. [2]Cisplatin + etoposide × 1 cycle followed by cisplatin + irinotecan × 3 cycles. LUL, left upper lobectomy; RLL, right lower lobectomy; RM/LL, right middle and lower lobectomy; RUL, right upper lobectomy.

ethylenediaminetetraacetic acid buffer (pH 8.0). Sections stained for chromogranin A (1:500, polyclonal; DAKO), synaptophysin (1:100, polyclonal; DAKO), neural cell adhesion molecule (NCAM) (1:200, Lu243; Nihon Kayaku, Tokyo, Japan), thyroid transcription factor (TTF)-1 (1:100, 8G7G3/1; DAKO), p63 (1:100, 4A4; DAKO) and D2-40 (1:50, D2-40; DAKO) were treated with 0.02 mol/L citrate buffer (pH 6.0). The slides were incubated overnight with each primary antibody. Diaminobenzidine was used as the chromogen, and hematoxylin as the counterstain.

Positive staining was defined as distinct linear membrane staining for neural cell adhesion molecule, cytoplasmic staining for chromogranin A and synaptophysin, and nuclear staining for TTF-1 and p63. Immunostaining of each of the SCLC and non-SCLC components was graded on a scale of 0–3+ according to the percentage of positive tumor cells (0 = none; 1+ = 1–33%; 2+ = 34–66%; 3+ = 67–100%). We then carried out immunohistochemical identification of lymph vessels with or without tumor embolisms for a representative slide.[22] After independent evaluation by two of us (T. F. and K. T.), judgment consensus was obtained by joint viewing of the slides using a multiheaded microscope.

Analysis of *EGFR* mutational status. In our previous study, we established a practical and precise non-sequencing method for detecting *EGFR* mutations involving high-resolution melting analysis (HRMA) using LCGreen I dye (Idaho Technology, Salt Lake City, UT, USA).[23] First we screened for the *EGFR* mutations, DEL and L858R, using the HRMA method in formalin-fixed paraffin sections obtained from surgically resected combined SCLC with Ad. Human genomic DNA (Roche Diagnostics, Basel, Switzerland) was used as a control sample with wild-type *EGFR*. Second, we used 10% formalin-fixed, paraffin-embedded surgical specimens of primary combined SCLC from patients demonstrating DEL or L858R by HRMA, and the DNA was extracted from each of the SCLC and Ad components, respectively, the areas of which were clearly determined morphologically after laser capture microdissection (Arcturus Engineering, Mountain View, CA, USA) of the tumor tissue.[24] Nested polymerase chain reaction (PCR) was carried out to amplify exons 19 and 21 of *EGFR* using previously described primers.[17] The PCR products were electrophoresed on 2% agarose gels and subcloned into the TA vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA), then the sequences were determined with M13 primers using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Results

Clinical characteristics. The clinical characteristics of the six patients are shown in Table 2. All patients were Japanese, aged between 62 and 77 years (mean 71.7 years). Five patients were male and one was female. Five patients were smokers whereas the remaining patient had never smoked. The median survival time of the six patients was 16.8 months (range 0.4–27.4 months); one patient died of heart failure 13 days after left pneumonectomy.

All six tumors were located in the peripheral portion of the lung. On clinical evaluation, three patients were staged as Ia (T1N0M0), one as Ib (T2N0M0) and two as IIb (T2N1M0). Preoperative pathological diagnoses were obtained in three patients and comprised one case each of SCLC, non-SCLC and Ad.

Pathological findings. Among six patients with combined SCLC with Ad, histological examination demonstrated that four had

Table 3. Histological findings of primary tumor and lymph node metastases, and epidermal growth factor receptor (*EGFR*) mutation

Patient no.	Stage (pTNM)	Ratio of each component (%)			Histological type of lymph node metastasis		BAC-like extension	*EGFR* mutation
		SCLC	Ad	Sq	Mediastinal	Hilar		
1	IIa (110)	95	5	0	Non†	SCLC	Absent	Wild type
2	IIIa (220)	80	10	10	SCLC	SCLC	Present	Wild type
3	IIIb (410)	70	30	0	Non†	Ad	Present	L858R
4	IIIb (420)	55	45	0	Ad	SCLC or Ad‡	Present	Wild type
5	IIIa (220)	35	60	5	Ad	SCLC or Ad‡	Present	Wild type
6	Ib (200)	5	95	0	Non†	Non†	Present	Wild type

†The patient had no mediastinal or hilar lymph node metastasis. ‡The patient had lymph node metastasis only from the SCLC component, and another lymph node showing metastasis only from the Ad component. Ad, adenocarcinoma; BAC, bronchioloalveolar carcinoma; hilar, hilar lymph node; L858R, mutation at codon 858 of *EGFR*; medical, mediastinum lymph node; pTNM, pathological TNM; SCLC, small cell lung carcinoma; Sq, squamous cell carcinoma.

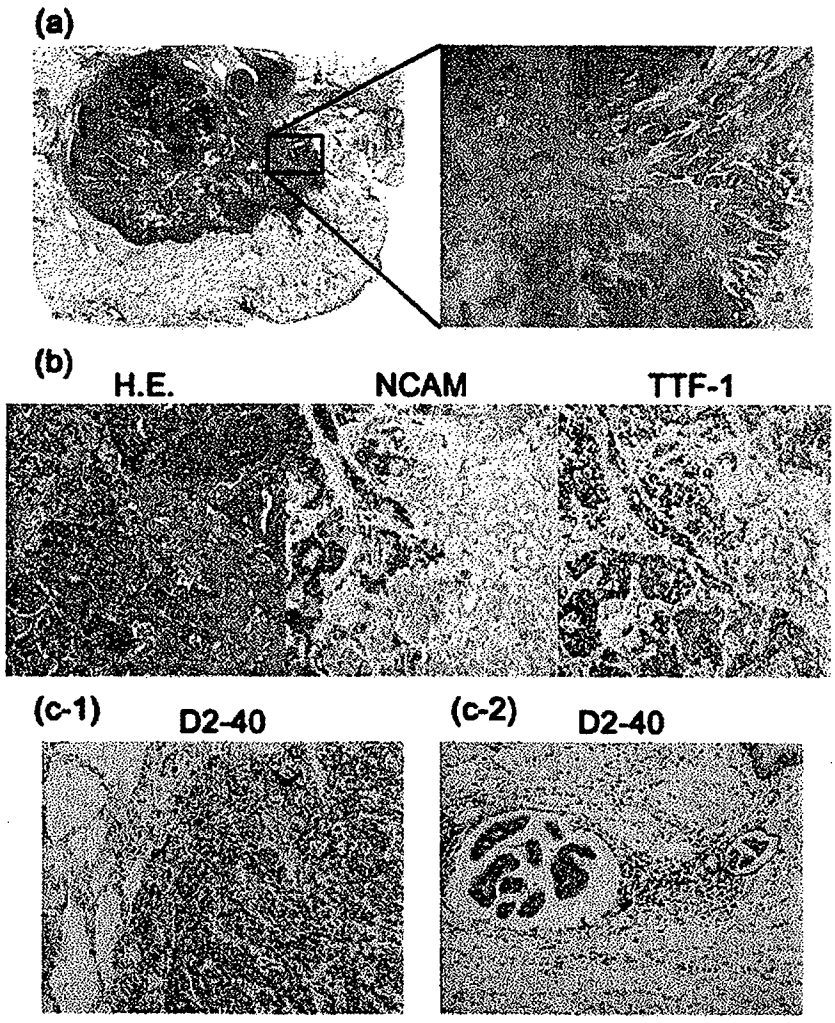

Fig. 1. Combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad). (a) The periphery of this tumor consisted of a non-mucinous bronchioloalveolar carcinoma-like extension (patient no. 3). (b) The transitional zone between the SCLC and Ad components had poorly differentiated cells, shown by the immunohistochemical studies (patient no. 1). (c) D2-40 with a membranous staining pattern of the lymph vessels. Tumor embolism of lymph vessels was confirmed by D2-40 staining (patient no. 3). (c-1) SCLC cell embolisms increased in number around the primary lesion. (c-2) Ad cell embolisms invaded the lymph vessels.

SCLC combined only with an Ad component (ratio of Ad in the tumor: 5, 30, 45 and 95%), whereas two had both Ad and Sq components (ratio of Ad/Sq: 10%/10% and 60%/5%, respectively). On pathological staging, one patient was staged as Ib (T2N0M0), one as IIa (T1N1M0), two as IIIa (T2N2M0) and two as IIIb (T4N1M0 and T4N2M0). In five of the six patients, the Ad components were observed in the peripheral part of the tumor showing a lepidic extension pattern, simulating bronchioloalveolar carcinoma. In the remaining one patient, Ad formed a minor component comprising approximately 5% of the tumor (Table 3). The Ad components in two patients showed a micropapillary growth pattern, whereas mucin production was not detected in any patient (Fig. 1a). The boundary between the SCLC and Ad components was not clear, and showed an

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indeterminate component that suggested gradual morphological transition from one to the other (Fig. 1b). In the two patients who also had combined Sq, the Sq component showed keratinization and was distinct from the SCLC component, but the border between the Ad and Sq components was unclear.

The results of immunohistochemical studies carried out in five cases are shown in Table 1. The specimen from patient no. 6 was not available. All of the SCLC components showed positive staining for at least one neuroendocrine marker. In addition, the Ad components in all five patients examined showed positive staining for at least one neuroendocrine marker, although semiquantitative assessments of the percentage of positive Ad cells were lower than those for SCLC cells in the same tumor. Also, the Ad components showed positive staining for TTF-1 in all five patients. TTF-1 staining of the SCLC component tended to be similar to that of the Ad component in terms of the percentage of positive cells. p63 immunostaining served as a good marker of Sq differentiation.

Status of lymph node metastasis. Five patients had pathologically confirmed hilar lymph node metastases, and three of them also had histologically proven mediastinal lymph node metastases, which had not been evident at the time of preoperative clinical evaluation (Table 3). Among these five patients with hilar lymph node metastases, two showed only SCLC in the metastatic lesion, one showed Ad only, and two showed SCLC or an Ad component that had developed separately in each lymph node. Among the three patients with mediastinal lymph node metastases, one had only SCLC in the nodes, and two had an Ad component only. Metastatic Ad components were found only in patients with a primary tumor in which Ad accounted for more than 30% of the total volume.

In the six patients, we identified tumor embolism of the lymph vessels immunohistochemically with D2-40 staining. There were approximately 800–1000 lymph vessels in each of these tumors per representative slide. The major component invading the lymph vessels around the tumors was SCLC cells. Even in the two patients who had mediastinal lymph node metastases with an Ad component, the SCLC cells tended to spread to the lymph vessels rather than the Ad cells (Table 1).

***EGFR* mutational status.** First, we analyzed 10 surgically resected samples from six patients with combined SCLC and Ad by HRMA. Analysis of exon 19 demonstrated curves identical to those of the control (wild type) in all samples, as shown in Fig. 2a. In the analysis of exon 21, thorough melting curves were obtained for two samples from patient no. 3, showing a different curve from the control, whereas the other eight samples demonstrated curves identical to the control (wild type), as shown in Fig. 2b. The normal lung tissue from patient no. 3, who was a female non-smoker, showed a wild-type curve, and therefore we judged that this patient had L858R in exon 21 of *EGFR*.

Next we confirmed this mutation in the SCLC and Ad components in patient no. 3. DNA was extracted from each SCLC and Ad component separately using laser capture microdissection or by manual microdissection, which was carried out for each clearly determined component on paraffin-embedded sections. Sequence analysis of subcloned PCR products obtained from the separate components was carried out. Examination of both SCLC and Ad components showed an identical mutation (L858R) in exon 21 (Fig. 3), confirming the results obtained by HRMA.

Discussion

The present study using microdissected tumor tissue is the first to report a patient with combined SCLC with Ad showing the *EGFR* mutation in both the SCLC and Ad components. *EGFR* mutations, especially DEL and L858R, have been reported in

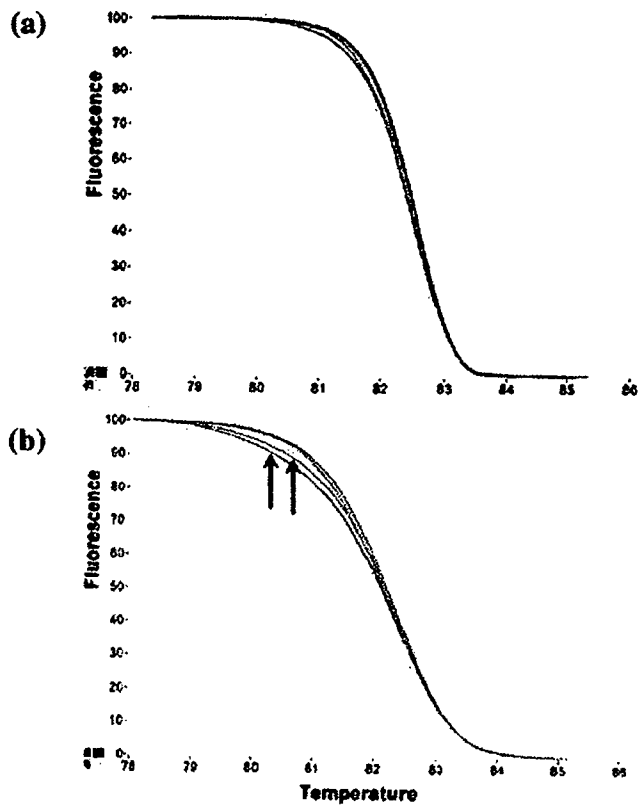

Fig. 2. Results of high-resolution melting analysis (HRMA). Adjusted melting curves obtained by HRMA of combined small cell lung carcinoma (SCLC) with primers designed to detect mutations in (a) exon 19 or (b) exon 21 of epidermal growth factor receptor (*EGFR*). Two samples from patient no. 3 were identified as containing the L858R mutations (↑). The DNA extracted from normal lung tissue of patient no. 3 was identified as wild type (not shown).

Ad of the lung. These somatic mutations in the kinase domain of *EGFR* have been shown to be predictive molecular markers for sensitivity to kinase inhibitors such as gefitinib (Iressa; AstraZeneca, Osaka, Japan). However, these mutations have rarely been demonstrated in SCLC. To our knowledge, there have been two reported cases of metastatic SCLC harboring DEL in exon 19 of *EGFR* showing responsiveness to EGFR tyrosine kinase inhibitors.[19,20,25] Considering that the diagnosis of SCLC is often based on small biopsy specimens that may not be sufficiently representative of the total tumor, there is a possibility that any combined component may be overlooked.

In a clinical setting, the distinction of SCLC from non-SCLC is critical because of major differences in management and prognosis between the two cancers. SCLC is well known to be more common in men and smokers, but so far SCLC with *EGFR* mutations has been detected only in female patients who have never smoked,[19,20] as was the case in our present female patient. Thus it seems reasonable to suggest that in clinically unusual SCLC patients, for example those who are non-smokers and female, showing peripheral nodular lesions and histological combination with Ad, *EGFR* mutation status should be analyzed because previous studies have shown that EGFR tyrosine kinase inhibitors are effective in patients with metastatic SCLC with *EGFR* mutations.

The present study is considerably informative with regard to the origin and histogenesis of SCLC. *EGFR* mutation is detected in patients with pre-invasive adenocarcinomatous lesions such as atypical adenomatous hyperplasia and bronchioloalveolar

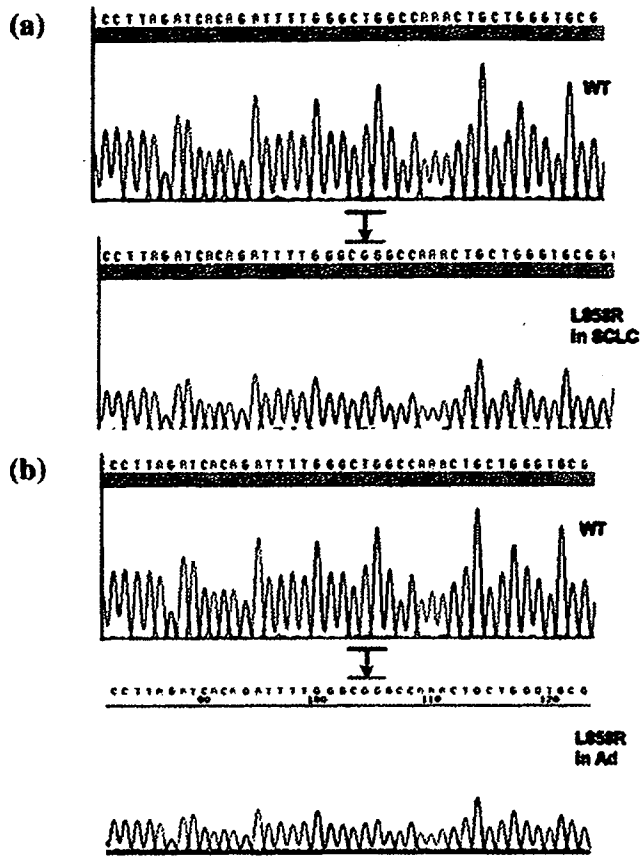

Fig. 3. Results of DNA sequencing from patient no. 3. The tumor of patient no. 3 was microdissected into the small cell lung carcinoma (SCLC) and adenocarcinoma (Ad) components. (a) Sequence analysis of the subcloned polymerase chain reaction (PCR) products from the microdissected SCLC component. (b) Sequence analysis of the subcloned PCR products from the microdissected Ad component. The patient had a tumor with L858R of EGFR, which was in both the SCLC and Ad components.

carcinoma, which eventually progress to invasive lung Ad.[26] In addition, *EGFR* mutations are also linked to Ad with a bronchioloalveolar carcinoma component.[27] Thus it is suggested that *EGFR* mutation occurs and plays a critical role in the early developmental stage of lung Ad. The mutation is detected more frequently in Ad in female non-smokers than in male smokers. In the present study, the only patient with SCLC harboring an *EGFR* mutation was female and a non-smoker, and the combined Ad component also harbored the same mutation. Moreover, as mentioned above, the two SCLC patients with *EGFR* mutation reported previously were also female and non-smokers. These findings imply that the mutations are an early genetic event in carcinogenesis of the lung and at least a certain proportion of SCLC may originate as a result of progression or transformation of Ad harboring *EGFR* mutation.

This phenomenon can also be linked to pathological features. The histological patterns of lymph node involvement showed that Ad components spread to mediastinal lymph nodes in the patients with hilar lymph node involved by SCLC or Ad component. Considering the status of tumor embolism of the lymph vessels observed using D2-40 staining, SCLC cell embolisms, but not Ad, increase in number around primary lesion in these tumors. It is suggested that a common uncommitted stem cell might differentiate into each component after involvement in a lymph node. Furthermore, positive staining for TTF-1, which is a highly specific immunohistochemical marker identifying carcinomas of pulmonary origin (especially non-mucinous Ad and SCLC),[28] was shown in the SCLC and Ad components, but not Sq. Previous studies have demonstrated TTF-1 expression in 83–100% of SCLC, but low expression in Sq.[29,30] These findings could be interpreted as being compatible with the hypothesis that SCLC and Ad originate from a common uncommitted stem (or precursor) cell originally expressing TTF-1.[31] It is possible to postulate that a fraction of SCLC possessing stem (or precursor) cell properties might have the potential to form an Ad component. In fact, in the present cases, there were some areas comprising morphologically indeterminate tumor cell components at the border of the SCLC and Ad components.

The rarity of patients with combined SCLC makes it difficult to determine the optimal management and biological characteristics of this tumor. However, the present findings suggest that the classical classification of lung cancer might provide insufficient management for a specified subpopulation in molecular targeted therapy. Although this retrospective study examined only a very limited number of lung carcinoma cases, we consider that the findings provide useful information for understanding the biology of this lung cancer and devising more effective forms of clinical management.

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Epidermal growth factor receptor mutation, but not sex and smoking, is independently associated with favorable prognosis of gefitinib-treated patients with lung adenocarcinoma

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Epidermal growth factor receptor (*EGFR*) mutations have been reported as a predictive factor for favorable prognosis of gefitinib-treated patients with lung adenocarcinoma. However, its confounding with sex and smoking makes it unclear whether the *EGFR* mutation is independently associated with prolonged patient survival. In this study, we analyzed a large-scale database to discriminate the survival impact of *EGFR* mutations against those of sex and smoking after gefitinib therapy. *EGFR* mutations in exon19 and exon21 named drug-sensitive *EGFR* mutations were examined to investigate the impact of *EGFR* mutation, sex, and smoking status on survival of 362 gefitinib-treated patients with lung adenocarcinoma. Drug-sensitive *EGFR* mutations were detected in 169 patients (46.7%). The multivariate analysis including *EGFR*, sex, and smoking status showed that drug-sensitive *EGFR* mutations were significantly related to longer overall survival (OS) ($P < 0.001$) and progression-free survival (PFS) ($P < 0.001$). In addition, we investigated the impact of sex and smoking status according to *EGFR* mutation status, and the impact of *EGFR* mutation status according to sex and smoking status on survival. Sex and smoking status were not significantly associated with longer OS and PFS according to *EGFR* mutation status. Drug-sensitive *EGFR* mutations were significantly associated with longer OS and PFS according to sex or smoking status. Our results indicated that drug-sensitive *EGFR* mutations were the only independent factor for longer survival of patients treated with gefitinib, suggesting that patient selection based on *EGFR* mutation status for gefitinib therapy will lead to a better outcome for patients with lung adenocarcinoma. (*Cancer Sci* 2008; 99: 303–308)

Epidermal growth factor receptor (*EGFR*) is a receptor tyrosine kinase that is highly expressed in cancer cells.[1] Gefitinib and erlotinib are reversible EGFR tyrosine kinase inhibitors (EGFR-TKI) used for the treatment of non-small-cell lung cancer (NSCLC) patients.[2] Previous studies have focused on identifying factors that are useful indicators when selecting candidate patients for gefitinib treatment. An adenocarcinoma histology, female sex, a never-smoking status, and an East Asian ethnicity are clinicopathological factors that are associated with sensitivity to gefitinib and these factors can be indicators for gefitinib therapy.[2,3]

Mutations in *EGFR* have been reported, particularly in NSCLC.[1,4,5] *EGFR* mutations are frequently located in exon19 and exon21 of the *EGFR* tyrosine kinase domain and play an oncogenic role in adenocarcinoma.[6] Previous studies have shown that a positive *EGFR* mutation status is significantly related to adenocarcinoma histology, female sex, a never-smoking status, and an East Asian ethnicity.[4,5,7–10] Of clinical interest, approximately 80% of patients with the *EGFR* mutation, especially exon19 deletions and the L858R mutation at exon21, are associated with sensitivity to reversible EGFR-TKIs. Positive *EGFR* mutation status is considered to predict a favorable clinical outcome for NSCLC patients treated with gefitinib, especially those from East Asia.[11–13] However, Cappuzzo *et al.* reported that *EGFR* and *HER2* gene copy numbers, but not *EGFR* mutations, were predictors for good clinical outcome of reversible EGFR-TKI therapy, especially in white populations,[14,15] but these facts have not been confirmed in patients of East Asia origin.[16–18]

Although *EGFR* mutations could be a predictor for good clinical outcome of reversible EGFR-TKI therapy, the impact of *EGFR* mutation, sex, and smoking status on patient prognosis of gefitinib treatment is still an issue of interest. Initially, female sex, never-smoking status, and adenocarcinoma histology were identified as predictive factors for having *EGFR* mutations,[4,5,7,10] therefore, in identifying predictive factors on survival after gefitinib therapy, *EGFR* mutation strongly confounds with sex, smoking status, and histology. In addition, recent reviews suggested female sex and never-smoking status as prognostic factors of NSCLCs.[19,20] Some studies have suggested that smoking status can be a useful indicator when selecting candidate patients for gefitinib treatment.[3,21] Thus, it is important to discriminate the survival impact of *EGFR* mutation against those of clinicopathological factors in patients treated with gefitinib.

To address these matters, we combined data from three institutions in Japan to establish a large-scale database and evaluated the factors that were related to clinical outcome of lung adenocarcinoma patients treated with gefitinib.

Materials and Methods

Patients and gefitinib treatment. We collected the data (clinical records and *EGFR* mutation status) of 408 NSCLC patients treated with gefitinib from the National Cancer Center Hospital (Tokyo, Japan; 207 patients), Aichi Cancer Center Hospital

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Table 1. **Patient characteristics and drug-sensitive epidermal growth factor receptor (*EGFR*) mutation in 408 non-small-cell lung cancer patients treated with gefitinib**

Variables	No. of patients	*EGFR* mutation (%)
Age		
<64 years	177	84 (47.5)
≥64 years	185	85 (45.9)
Sex		
Female	162	101 (62.3)
Male	200	68 (34.0)
Smoking history		
Never	170	104 (61.2)
Ever	192	65 (33.9)
0 < PY < 20	40	28 (65.0)
20 ≤ PY	152	37 (25.7)
Disease stage		
Rec	220	120 (54.5)
Adv	142	49 (34.5)
Institution		
NCC	189	83 (43.9)
ACC	91	54 (59.3)
Okayama	82	32 (39.0)

ACC, Aichi Cancer Center Hospital, Nagoya, Japan; Adv, advanced disease; *EGFR* mutation, drug-sensitive *EGFR* mutation; NCC, National Cancer Center Hospital, Tokyo, Japan; Okayama, Okayama University Hospital, Okayama, Japan; PY, pack-years; Rec, recurrent disease;

(Nagoya, Japan; 103 patients), and Okayama University Hospital (Okayama, Japan) with NHO Sanyo National Hospital (Yamaguchi, Japan; 98 patients). These cases were independently analyzed in each institution and 364 of them had been previously reported.[11,13,16,22] An additional 44 patients from Aichi Cancer Center Hospital were included in the present study. Because the majority of our patients had an adenocarcinoma histology (362 [88.7%] of 408 patients), we limited our analyses to patients with adenocarcinoma histology. Total patients consisted of: 162 (44.8%) female and 200 (55.2%) male; 170 (47.0%) never-smokers and 192 (53.0%) ever-smokers; and 220 (60.8%) recurrent disease and 142 (39.2%) advanced disease. Never-smokers were those with lifetime exposure of 100 cigarettes or less, ever-smokers were those with lifetime exposure of more than 100 cigarettes. Ever-smokers were classified into two categories based on the degree of cumulative smoking dose: 40 patients with light- to moderate-smoking status (0 < pack-years [PY] < 20), and 152 patients with heavy-smoking status (20 ≤ PY).[23] The details of the patient characteristics are shown in Table 1. All patients had initiated gefitinib treatment (250 mg/day) between November 2000 and August 2006 in each institution. In the majority of patients, gefitinib treatment was continued as long as possible until disease progression, development of unacceptable toxicity, or patients' refusal to continue treatment. Institutional review board permission and informed consent were obtained for all patients at each institution. The permission numbers of the institutional review boards related to the present study at each institution are as follows: G12-06 for National Cancer Center Hospital; No.19-12 for Aichi Cancer Center Hospital; No.1 and No.48 for Okayama University Hospital; and No.0306 for NHO Sanyo National Hospital.

Detection of *EGFR* mutations. The direct sequence with genomic DNA was used for all specimens analyzed in Okayama University Hospital and 66 specimens in National Cancer Center Hospital.[9,13] High-resolution melting analysis was used to detect exon19 deletion and mutations in exon21 in 141 specimens from National Cancer Center Hospital.[22,24] The RNA-based analysis for 59 specimens using one-step reverse transcription–polymerase chain reaction for exons 18–21 and for 44 specimens using polymerase chain reaction-based assay for exons 19 and 21 was carried out at Aichi Cancer Center Hospital.[8,25] Limited to adenocarcinoma, our 362 patients consisted of 189 patients from National Cancer Center Hospital, 91 from Aichi Cancer Center Hospital, and 82 from Okayama University Hospital. For this study, drug-sensitive *EGFR* mutations, exon19 deletion or insertion, and exon21 L858R or L861Q mutation were considered as cases with the drug-sensitive *EGFR* mutation, and others were considered as the *EGFR* wild-type.

Statistical analyses. In this study, the impact of *EGFR* mutation status on survival after gefitinib treatment was examined considering smoking and sex status as confounder or effect modifier. For this purpose, we chose overall survival (OS) and progression-free survival (PFS) as endpoints. The OS and PFS were calculated from start of gefitinib treatment until the date of death or the last follow-up for OS and until confirmed disease progression or death for PFS. The Kaplan–Meier method was applied to estimate OS and PFS. Differences in OS and PFS among groups were assessed by log–rank test. Univariable and multivariable Cox proportional hazard models combined with stratification were applied to further evaluate significance of examined factors on OS and PFS. In the multivariate model, confounders considered were age as a continuous variable, disease stage (recurrence or advanced), and institutions. Smoking status was examined in the models using cumulative exposure to smoking (PY). When necessary, χ^2-tests were applied to examine differences in categorical factors across groups. The multivariate logistic regression model was used to identify baseline factors that might independently predict for the presence of drug-sensitive *EGFR* mutations. Statistical analyses were carried out using StatView 5.0 Program for Windows (SAS Institute, Cary, NC). All statistical tests were two-sided and *P*-values less than 0.05 were defined as being statistically significant.

Results

***EGFR* mutation and clinicopathological factors.** Drug-sensitive *EGFR* mutations were present in 169 (46.7%) of 362 patients and were comprised of 95 mutations in exon19 (93 deletions and two insertions) and 74 mutations (73 L858R and one L861Q) in exon21. The relationships between the *EGFR* mutation status and clinical factors are shown in Table 1. Multivariate analysis indicated that sex ($P = 0.045$), smoking status ($P < 0.001$), and disease status ($P = 0.002$), but not institutional difference, were significantly related to drug-sensitive *EGFR* mutations.

Impact of *EGFR*, sex, and smoking dose on survival of patient. Two hundred and sixty-three patients died, and the median follow-up period for the 99 survivors was 26.5 months (range, 1.6–58.5 months). The median survival time or the median progression-free survival time of all the patients was 15.3 and 4.0 months, respectively. The survival of patients was examined according to their *EGFR* mutation status, sex status, and smoking dose (PY = 0/0 < PY < 20/20 ≤ PY). The OS and PFS periods of patients with the drug-sensitive *EGFR* mutation and female sex status were significantly longer than those with *EGFR* wild-type (OS: $P < 0.001$; PFS: $P < 0.001$) and male sex status (OS, $P = 0.017$; PFS, $P = 0.002$) (Table 2). The OS and PFS periods of the patients were significantly related to the degree of smoking in a dose-dependent manner (OS, trend $P = 0.002$; PFS, trend $P < 0.001$) (Table 2). To further evaluate cumulative exposure to smoking, we explored two other thresholds, PY30 and PY40, similar to the PY20 analysis. As all of the analyses showed consistent trends (Supplementary Materials), we chose the commonly accepted threshold PY20 in the further analyses.[23] Multivariate analysis including *EGFR*, sex, and smoking status

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Table 2. Univariate and multivariate Cox proportional hazard models to further evaluate significance of drug-sensitive epidermal growth factor receptor (*EGFR*) mutation, sex, and smoking status on overall survival (OS) and progression-free survival (PFS) in 408 non-small-cell lung cancer patients treated with gefitinib

Co-variates	OS				PFS			
	Univariate	*P*	Multivariate†	*P*	Univariate	*P*	Multivariate†	*P*
EGFR (Mut *versus* Wt)	0.43 (0.33–0.55)	<0.001	0.48 (0.36–0.63)	<0.001	0.28 (0.22–0.35)	<0.001	0.29 (0.22–0.37)	<0.001
Sex (male *versus* female)	0.74 (0.58–0.95)	0.017	1.00 (0.71–1.40)	0.980	0.74 (0.58–0.95)	0.002	1.06 (0.78–1.45)	0.710
Smoking								
Ever PY ≥ 20	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
Ever PY < 20	0.67 (0.47–1.00)	0.050	0.95 (0.62–1.46)	0.810	0.70 (0.49–1.02)	0.061	1.09 (0.74–1.61)	0.660
Never	0.64 (0.50–0.83)	<0.001	0.83 (0.58–1.20)	0.330	0.57 (0.45–0.72)	<0.001	0.76 (0.54–1.07)	0.120
	Trend $P = 0.002$		Trend $P = 0.61$		Trend $P < 0.001$		Trend $P = 0.15$	

†Adjusted for age, disease stage, institution, and three variables of interest (*EGFR*, sex, and smoking status). –, not applicable; Mut, drug-sensitive *EGFR* mutation; PY, pack-years; Wt, *EGFR* wild-type.

Table 3. Impact of sex and smoking according to drug-sensitive epidermal growth factor receptor (*EGFR*) mutation status on overall survival (OS) and progression-free survival (PFS) in 408 non-small-cell lung cancer patients treated with gefitinib

Co-variates	OS				PFS			
	Univariate	*P*	Multivariate†	*P*	Univariate	*P*	Multivariate†	*P*
EGFR (Mut)								
Sex (male *versus* female)	1.05 (0.70–1.56)	0.83	1.18 (0.71–1.96)	0.52	1.05 (0.70–1.56)	0.83	1.27 (0.81–1.98)	0.30
Smoking								
Ever PY ≥ 20	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
Ever PY < 20	1.03 (0.55–1.92)	0.94	0.97 (0.49–1.89)	0.92	1.16 (0.67–2.00)	0.59	1.17 (0.65–2.10)	0.60
Never	0.94 (0.58–1.54)	0.82	0.85 (0.46–1.58)	0.61	0.84 (0.56–1.25)	0.38	0.79 (0.46–1.35)	0.39
	Trend $P = 0.94$		Trend $P = 0.84$		Trend $P = 0.34$		Trend $P = 0.32$	
EGFR (Wt)								
Sex (male *versus* female)	0.82 (0.59–1.14)	0.23	0.94 (0.60–1.47)	0.78	0.86 (0.63–1.17)	0.33	0.87 (0.56–1.35)	0.53
Smoking								
Ever PY ≥ 20	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
Ever PY < 20	0.85 (0.46–1.59)	0.62	0.95 (0.54–1.69)	0.86	1.20 (0.69–2.10)	0.52	1.31 (0.74–2.30)	0.35
Never	0.79 (0.57–1.11)	0.17	0.93 (0.59–1.47)	0.76	0.92 (0.68–1.26)	0.61	0.98 (0.62–1.55)	0.93
	Trend $P = 0.38$		Trend $P = 0.95$		Trend $P = 0.66$		Trend $P = 0.63$	

†Adjusted for age, disease stage, institution, and two variables of interest (sex and smoking status). –, not applicable; Mut, drug-sensitive *EGFR* mutation; PY, pack-years; Wt, *EGFR* wild-type.

showed that only drug-sensitive *EGFR* mutations were significantly associated with longer OS and PFS (OS: hazard ratio = 0.48, 95% CI = 0.36–0.63, $P < 0.001$) (PFS: hazard ratio = 0.29, 95% CI = 0.22–0.37, $P < 0.001$) (Table 2).

Next, we separated the impact of *EGFR*, sex, and smoking status on survival of patients treated with gefitinib to further understanding of these factors. For this purpose, we carried out two analyses focused on the following issues: the impact of sex and smoking status, according to *EGFR* mutation status, on survival (Table 3); and the impact of *EGFR* mutation status, according to sex and smoking status, on survival (Table 4). Table 3 shows that sex and smoking status were not significantly associated with longer OS and PFS among patients with the same *EGFR* mutation status (drug-sensitive *EGFR* mutation/ *EGFR* wild-type) using univariate and multivariate analyses. Kaplan–Meier plots stratified according to sex and *EGFR* mutation status and according to smoking dose and *EGFR* mutation status are shown in Fig. 1. Regarding the impact of *EGFR* mutation status, according to sex and smoking status, on survival, drug-sensitive *EGFR* mutations were significantly related to longer OS and PFS among groups of the same sex and smoking status, as shown in Table 4.

Taken together, our results clearly indicate that drug-sensitive *EGFR* mutations are the only independent factor for favorable prognosis of patients treated with gefitinib.

Discussion

Previously, we independently reported a relationship between the positive *EGFR* mutation status and clinical benefit in Japanese patients treated with gefitinib.[11,13,16,22] Analyses of a large-scale database might not only be useful to confirm or explore factors for the favorable clinical outcome with gefitinib, but also to improve our understanding of the impact of various factors on gefitinib treatment for patients. For this purpose, we combined our data and re-analyzed the factors that were assumed to affect clinical outcomes among gefitinib-treated patients with lung adenocarcinoma. Our study indicated that drug-sensitive *EGFR* mutation status was a significant factor of longer survival among gefitinib-treated patients, although sex and smoking status were significantly associated with favorable prognosis in univariate analyses. Indeed, Han *et al.* also showed the advantage of *EGFR* mutations for gefitinib effect compared with clinical factors.[26] As a point of discussion, it has been controversial whether the *EGFR* mutation is a good prognostic factor of NSCLC or not.[27–29] One of the reasons for the discrepancy might be that *EGFR* status (wild- or mutant-type) might influence not only the natural prognosis (untreated) but also the outcome after treatment with some chemotherapeutic drugs.[30] Previous studies did not precisely analyze different kinds of chemotherapeutic drugs to estimate the prognosis of

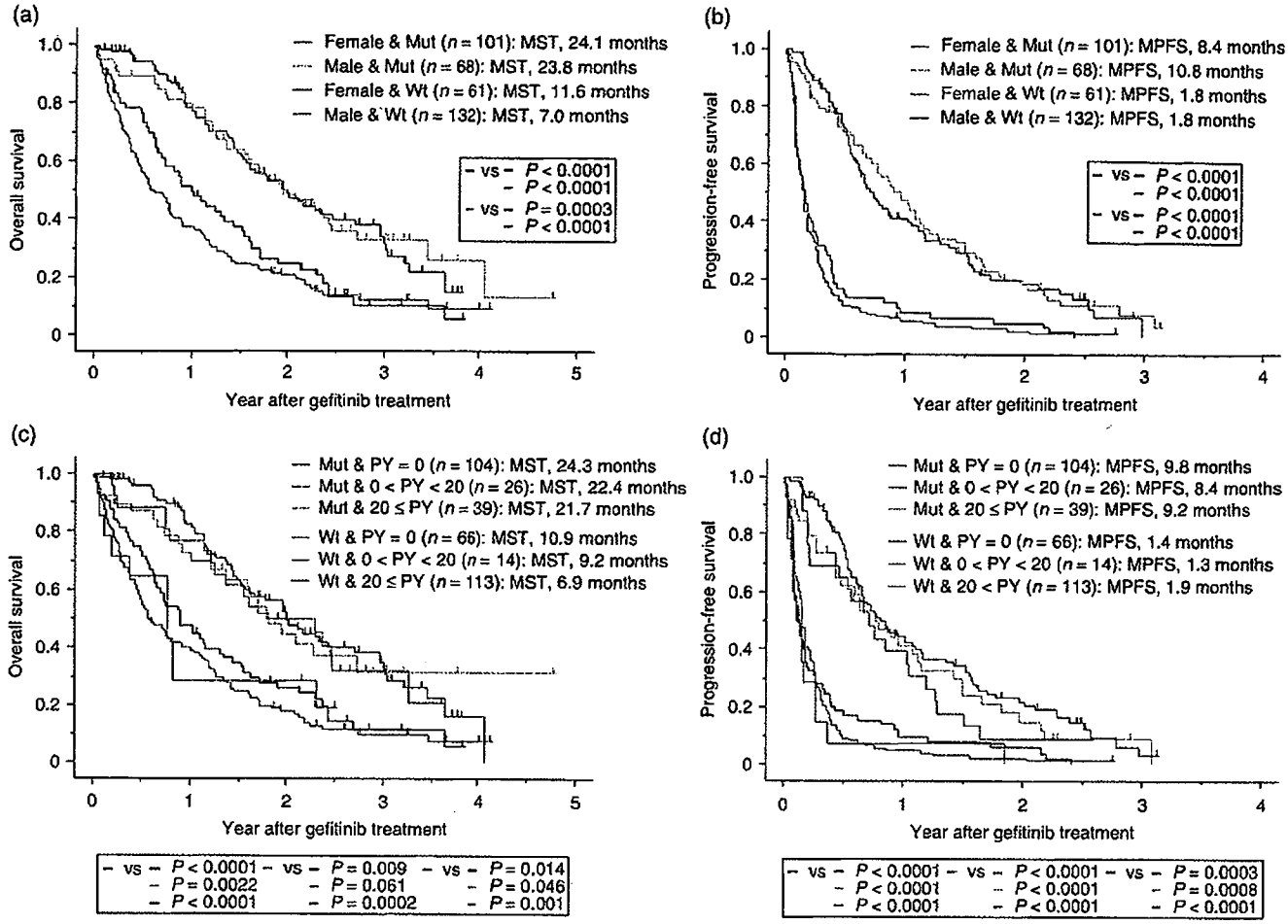

Fig. 1. Kaplan–Meier plot of survival times in 408 non-small-cell lung cancer patients treated with gefitinib. (a) Overall survival of patients classified into four groups according to epidermal growth factor receptor (*EGFR*) mutation and sex status. (b) Progression-free survival of patients classified into four groups according to *EGFR* mutation and sex status. (c) Overall survival of patients classified into six groups according to *EGFR* mutation and smoking dose status. (d) Progression-free survival of patients classified into six groups according to *EGFR* mutation and smoking dose status. Mut, drug-sensitive *EGFR* mutation; Wt, *EGFR* wild-type; PY, pack-years; MST, median survival time; MPFS, median progression-free survival time. *P*-values were calculated using the log-rank test.

Table 4. Impact of drug-sensitive epidermal growth factor receptor (*EGFR*) mutation status according to sex and smoking status on overall survival (OS) and progression-free survival (PFS) in 408 non-small-cell lung cancer patients treated with gefitinib

Co-variates	OS				PFS			
	Univariate	*P*	Multivariate†	*P*	Univariate	*P*	Multivariate†	*P*
Sex								
Male	0.43 (0.30–0.61)	<0.001	0.47 (0.32–0.69)	<0.001	0.27 (0.20–0.38)	<0.001	0.28 (0.18–0.39)	<0.001
Female	0.47 (0.32–0.68)	<0.001	0.50 (0.33–0.74)	<0.001	0.30 (0.21–0.42)	<0.001	0.30 (0.20–0.46)	<0.001
Smoking								
Ever PY ≥ 20	0.43 (0.27–0.68)	<0.001	0.44 (0.28–0.71)	<0.001	0.30(0.20–0.46)	<0.001	0.29 (0.19–0.45)	<0.001
Ever PY < 20	0.47 (0.22–1.01)	0.052	0.41 (0.15–1.01)	0.079	0.32 (0.16–0.65)	0.002	0.24 (0.09–0.61)	0.003
Never	0.45 (0.31–0.65)	<0.001	0.48 (0.32–0.71)	<0.001	0.28 (0.20–0.39)	<0.001	0.30 (0.20–0.43)	<0.001

†Adjusted for age, disease stage, and institution. PY, pack-years.

EGFR mutated/wild-type tumors. Further studies are necessary for this issue.

Our results, along with previous findings of the basic research, supported that the drug-sensitive *EGFR* mutation, but not sex or smoking status, was basically an appropriate target of gefitinib. Because of their biological features influencing the effect of gefitinib, drug-sensitive *EGFR* mutant tumors should be distinguished from *EGFR* wild-type tumors. Indeed, the IRESSA Survival Evaluation in Lung Cancer study, in which patients were not selected based on the *EGFR* mutation, did not indicate the significant survival benefit of gefitinib treatment.[31] Considering these facts, prospective studies to evaluate the

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benefit of reversible EGFR-TKIs should be designed based on *EGFR* mutation status, but not on clinical factors like smoking status. In this point, the selection criteria of the First Line IRESSA Versus Carboplatin/Paclitaxel in Asia trial, which includes smoking status but not *EGFR* mutations, is not essential to investigate the effect of gefitinib, although smoking status data are accessible. By contrast, the West Japan Thoracic Oncology Group (WJOG) has organized a randomized phase III trial of IRESSA versus cisplatin/docetaxel for patients with drug-sensitive EGFR mutations. In clinical practice, patients with *EGFR* mutations would be strongly recommended for gefitinib treatment keeping in mind that a positive *EGFR* mutation is not a perfect factor for favorable clinical outcomes.

In conclusion, our study showed that drug-sensitive *EGFR* mutation, but not sex or smoking status, is the superior factor for likely maximizing the therapeutic effect of gefitinib, indicating that drug-sensitive *EGFR* mutations, regardless of sex and smoking status, were an appropriate determinant for gefitinib treatment. Patient selection based on *EGFR* mutation status for reversible EGFR-TKI treatment will lead to better understanding of gefitinib therapy, as well as a better clinical outcome for patients with lung adenocarcinoma.

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Supplementary Materials

The following supplementary material is available for this article:

Table S1. Impact of different cumulative smoking dose on survival

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1349-7006.2008.00688.x
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Evaluation of the Recommended Dose and Efficacy of Amrubicin as Second- and Third-Line Chemotherapy for Small Cell Lung Cancer

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Introduction: This study was conducted to evaluate the recommended dose and activity of amrubicin (AMR) as second- or third-line chemotherapy for small-cell lung cancer (SCLC).

Methods: Small-cell lung cancer patients with measurable disease who had previously been treated with at least one platinum-based chemotherapy regimen and had an Eastern Cooperative Oncology Group performance status of 0–2 were eligible. Two groups of patients were selected: (1) a group to be treated with second-line chemotherapy and (2) a group to be treated with third-line chemotherapy. AMR was administered to both groups as a 5-minute daily intravenous injection at a dose of 40 or 35 mg/m^2 for three consecutive days every 3 weeks.

Results: Between March 2003 and June 2006, 27 patients (second-line, 40 mg/m^2: 13 patients; third-line, 40 mg/m^2: seven patients; and 35 mg/m^2: seven patients) were enrolled. Although the 40-mg/m^2 dose of AMR was feasible (one of 13 patients developed febrile neutropenia and four of 13 patients had grade 4 neutropenia) and effective (six of 13 patients had a partial response) in the second-line group, it produced unacceptable toxicity in a third-line setting (three of seven patients with grade 3 nonhematologic toxicities [febrile neutropenia in two patients and fatigue in one patient] and four of seven patients with grade 4 neutropenia). The 35-mg/m^2 dose of AMR had acceptable toxicity in the third-line group (one of seven patients with febrile neutropenia and one of seven had grade 4 neutropenia) and moderate efficacy (one of seven patients had a partial response and two of seven had stable disease).

Conclusions: AMR exhibits significant activity as second-line or third-line chemotherapy for small-cell lung cancer. The recommended dose is 40 mg/m^2 in a second-line setting and 35 mg/m^2 in a third-line setting.

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Approximately 15% of lung cancer patients have small-cell lung cancer (SCLC). Unlike other types of lung cancer, SCLC is one of the most chemosensitive solid tumors.[1] However, after remarkably successful induction therapy, most patients have a relapse within 2 years as a result of the emergence of drug-resistant tumor cells. Thus, long-term survival is extremely uncommon, and less than 25% of patients with limited-stage disease and less than 2% of patients with extensive-stage disease remain alive at 5 years.[2–4] The results of second-line chemotherapy against SCLC are quite disappointing, with relatively low response rates, brief remissions, and a short survival time. A new effective agent is needed to achieve better treatment results in patients with recurring or refractory SCLC.

Amrubicin hydrochloride is a totally synthetic 9-aminoanthracycline that is metabolically activated to amrubicinol by a liver enzyme. Amrubicin and amrubicinol inhibit DNA topoisomerase II and exert a cytotoxic effect by stabilizing a topoisomerase II–mediated cleavable complex. They are approximately one tenth weaker than doxorubicin as a DNA intercalator.[5,6] The catatonic activity of amrubicinol in vitro is 18 to 220 times more potent than that of its parent compound, amrubicin.[7]

Amrubicin has been reported to have shown more potent antitumor activity than the representative anthracycline doxorubicin in several human tumor xenografts implanted in nude mice and to have produced almost no cardiotoxicity.[8,9] Amrubicin at 45 mg/m^2 on days 1 to 3 every 3 weeks has been shown to be active against previously untreated SCLC, with an overall response rate of 78.8% and a median survival time (MST) of 11.0 months.[10] Onoda et al.[11] found that amrubicin 40 mg/m^2 had significant activity and acceptable toxicity in previously treated patients. However, Kato et al.[12] reported finding that amrubicin at 45 mg/m^2 not only had promising activity but severe and unacceptable toxicity in patients, similar to those in the Onoda et al. study. Most patients enrolled in both studies received amrubicin as second-line treatment.

Accordingly, the results of the previous studies are of value in considering amrubicin as a key agent for the treatment of SCLC, not only untreated cases but in previously treated cases. However, the recommended dose for previ-

ously treated patients has not been determined, especially in third-line or greater settings.

The purpose of this study was to evaluate the toxicity and efficacy of amrubicin as second- and third-line chemotherapy in SCLC patients in a clinical setting to determine the recommended dose.

PATIENTS AND METHODS

Patient Selection

The subjects of this study were 27 patients with previously treated SCLC between March 2003 and June 2006 at Shizuoka Cancer Center. The recruitment criteria were (1) history of at least one platinum-based chemotherapy regimen and confirmation of refractory or recurrent SCLC based on the results of the following examinations: chest radiograph, computed tomography of the chest and abdomen, and other procedures as indicated, including magnetic resonance imaging of the head and positron emission tomography or combined positron emission tomography/computed tomography; (2) age 75 years or younger; (3) performance status of 2 or less according to the Eastern Cooperative Oncology Group scale; (4) adequate bone marrow, hepatic, and renal function; (5) no other serious disease; (6) written informed consent.

Treatment Methods

The treatment schedule comprised a 5-minute intravenous infusion of amrubicin in 50 ml normal saline on days 1 to 3 every 3 weeks. Patients receiving second-line chemotherapy were treated with a dose of 40 mg/m^2. The first seven consecutive patients in the third-line group were treated with a dose of 40 mg/m^2, and the next seven patients were treated with a dose of 35 mg/m^2. Before the start of treatment, the patient had to have an absolute neutrophil count of 1500/mm^3 or more, a platelet count of 100,000/mm^3 or more, aspartate aminotransferase and alanine aminotransferase values less than three times the maximum value in the normal range, and total bilirubin and creatinine values less than 1.5 times the maximum value in the normal range. Treatment with granulocyte colony-stimulating factor was permitted as a therapeutic intervention but was not mandatory as a prophylactic agent against neutropenia as hematologic toxicity. Subsequent doses were modified based on hematologic and nonhematologic toxicities at the discretion of the physician in charge. Complete blood count and biochemistry examinations were repeated at least once per week after the initial evaluation.

Evaluation of Response and Toxicity

Adverse events were recorded and graded using the National Cancer Institute Common Toxicity Criteria, Version 3.0 grading system. Tumor response was classified in accordance with the Response Evaluation Criteria in Solid Tumors. Patients were evaluated to determine the stage of their disease before treatment and when their disease progressed or recurred by taking a complete medical history and performing a physical examination, chest radiography, computed tomography of the chest and abdomen, and other staging procedures, such as magnetic resonance imaging of the head, and positron emission tomography.

Limited disease was defined as disease confined to one hemithorax, including bilateral mediastinal and bilateral supraclavicular nodes, and extensive disease (ED) was defined as any involvement beyond these confines. Primary refractory disease was defined as recurrence during the first-line chemotherapy regimen or less than 8 weeks after completing the initial chemotherapy regimen, and sensitive disease was defined as recurrence more than 8 weeks after completion of the first-line chemotherapy.

Definition of Recommended Dose

The recommended dose of amrubicin was defined as the dose at which severe toxicity occurred in less than 33% of the patients treated. At least six patients were treated at each dose level. Severe toxicity was defined as grade 4 hematologic toxicity and grade 3 or higher nonhematologic toxicity including febrile neutropenia.

Statistical Methods

Kaplan-Meier plots were prepared for overall survival, and median values were calculated. Overall survival was measured from the first day of amrubicin treatment to the day of death or the day last seen alive (cutoff).

RESULTS

Patient Characteristics

Between March 2003 and June 2006, 27 patients with recurring or refractory disease were enrolled in this study. The characteristics of the patients are listed in Table 1. Four patients were women and 23 were men, and the patients' median age was 64 years (range, 53–74 years). At the start of the treatment one patient had limited disease and 26 patients had ED. All 27 patients had been treated with some form of chemotherapeutic regimen: 13 had received one previous regimen, 14 had received two previous regimens. All patients had been previously treated with some form of cisplatin- (23 patients) or carboplatin-based combination chemotherapy; 19 patients had received an irinotecan-containing regimen and one patient had received a topotecan-containing regimen.

TABLE 1. Patient Characteristics

Characteristic	
Age, yr, median (range)	64 (53–74)
Gender: male/female	23/4
PS: 0/1/2	2/21/4
Stage: limited disease/extended disease	1/26
No. of previous chemotherapy regimens: 1/2	13/14
Amrubicin dose, days 1–3	
Second-line: 40 mg/m^2	13
Third-line: 40/35 mg/m^2	7/7
Refractory/sensitive	8/19

PS, performance status.

TABLE 2. Toxicity by Dose

		ANC		Hb		PLT		FN	Other
	Pts	G3	G4	G3	G4	G4	G3	G3	G3
Second-line: 40 mg/m^2	13	2	4	0	0	2	0	1	1 Dyspnea
Third-line: 40 mg/m^2	7	0	4	1	0	2	1	2	1 Fatigue anorexia
Third-line: 35 mg/m^2	7	5	1	1	0	3	0	1	0

Pts, patients; ANC, absolute neutrophil count; Hb, hemoglobin; PLT, ●●●; FN, febrile neutropenia; G, grade.

Toxicity

The toxicity of the regimen is summarized in Table 2. Table 2 shows the worst toxicity level in each patient. Grade 4 neutropenia was observed in four (31%) of the 13 patients receiving second-line chemotherapy with 40 mg/m^2 of amrubicin, and four cases of grade 4 neutropenia (57%) and one case of grade 3 (14%) were observed among the seven patients receiving third-line chemotherapy. However, only one of the seven third-line patients who received the reduced dose of 35 mg/m^2 developed grade 4 neutropenia (14%). Febrile neutropenia was observed in one patient (8%) in the second-line group who received 40 mg/m^2, two patients (28%) in the third-line group who received 40 mg/m^2, and one patient (14%) in the third-line group who received 35 mg/m^2. All other hematologic toxicities were mild. Two cases (28%) of grade 3 nonhematologic toxicities other than febrile neutropenia were observed one in the second-line group and the other in third-line group.

Accordingly, the recommended dose of amrubicin for second-line treatment and third-line treatment was concluded to be 40 mg/m^2/day and 35 mg/m^2/day, respectively.

Response and Survival

No complete responses and eight partial responses were observed among the 27 patients (29%). A comparatively high response rate was achieved in the second-line chemotherapy group, with a response rate of 46% (six of 13 patients) in the group who received the 40-mg/m^2 dose. The patients in the third-line group who received the 40-mg/m^2 dose and the 35-mg/m^2 dose had a similar response rate: 14% (one of seven patients). The sensitive cases and refractory recurrence cases had response rates of 42% (3/7) and 25% (5/20), respectively. The overall median survival time (MST) and 1-year survival rate were 9.2 months and 33.3%, respectively (Figures 1 and 2).

DISCUSSION

The outlook for SCLC patients who receive second-line chemotherapy has been poor, and few single agents have been capable of producing a high response rate among patients with early recurrence or disease progression during treatment. The new agents that have been most extensively evaluated in SCLC are the topoisomerase I inhibitors irinotecan and topotecan. A recent randomized phase III trial demonstrated that single-agent topotecan was at least as effective as the three-drug combination of cyclophosphamide, doxorubicin, and vincristine in patients with disease progression at least 60 days after initial therapy. Topotecan yielded a response rate of 24.3% versus 18.3% for cyclophosphamide, doxorubicin, and vincristine, with an MST of 25.0 versus 24.7 weeks and improved symptom control.[13] Two studies of irinotecan in patients with refractory SCLC have been reported in Japan, and the response rate in both studies was high: 50% in 16 patients and 47% in 15 patients.[14,15] Irinotecan and topotecan have been recognized as key drugs in the second-line treatment of SCLC. A recent phase III study that compared irinotecan plus cisplatin with etoposide and cisplatin in patients with ED-SCLC revealed a superior median survival rate and a superior 2-year survival rate for the irinotecan plus cisplatin combination.[16] As a result, irinotecan plus cisplatin was established as one of the standard first-line regimens for SCLC in Japan. Thus, it has been necessary to search for effective drugs other than the topoisomerase I inhibitors for previously treated SCLC.

A response rate of 79% has been reported for amrubicin at a dose of 45 mg/m^2 on days 1 to 3 in chemotherapy-naive ED-SCLC patients in an intent-to-treat analysis.[10] Because of its very high activity as a single agent in untreated ED-SCLC patients, amrubicin has since been approved for SCLC in Japan in April 2002. Amrubicin has mainly been used for previously treated SCLC in clinical practice because of the higher response rate of untreated SCLC and an antitumor mechanism that differs from that of platinum and topoisomerase I inhibitors.

However, the recommended dose of amrubicin for previously treated SCLC was unknown.

The dose approved by the Japanese Ministry of Labor, Health, and Welfare is 45 mg/m^2 on days 1 to 3. Kato et al.[12] conducted a phase II study of amrubicin 45 mg/m^2 in previ-

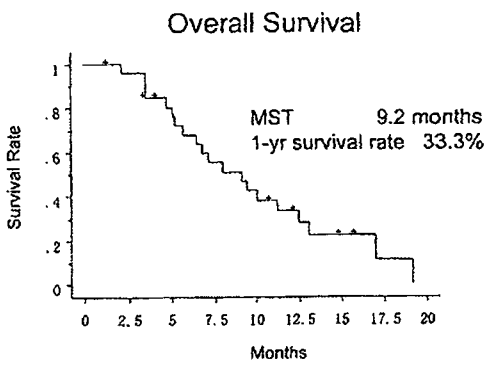

FIGURE 1. Kaplan-Meier plot of overall survival in all patients.

TABLE 3. Response by Dose

	No. of Patients	PR	SD	PD	RR (%)
Overall	27	8	8	11	29
Second-line: 40 mg/m^2	13	6	3	4	46
Third-line: 40 mg/m^2	7	1	3	3	14
Third-line: 35 mg/m^2	7	1	2	4	14
Sensitive	7	3	3	1	42
Refractory	20	5	5	10	25

PR, partial response; SD, stable disease; PD, progressive disease; RR, relative risk.

ously treated SCLC patients, mostly second-line chemotherapy patients, and reported severe hematologic toxicity and a high incident of febrile neutropenia.

The incidence of severe amrubicin toxicity at the 40-mg/m^2 dose as second-line chemotherapy was 31% (four of 13: grade 4 neutropenia in two, grade 4 neutropenia and febrile neutropenia in one, grade 4 neutropenia and grade 3 dyspnea in one), and this dose was acceptable. These results are similar to those reported in another study.[11] However, amrubicin 40 mg/m^2 induced severe toxicity in 57% of the third-line chemotherapy patients (4/7: grade 4 neutropenia in 2, grade 4 neutropenia and febrile neutropenia in 1, grade 4 neutropenia, febrile neutropenia and grade 3 fatigue in one). However, the rate of severe toxicity (14%: one of seven patients with grade 4 neutropenia and febrile neutropenia) at the lower dose (35 mg/m^2) of amrubicin was acceptable (Table 3).

The MST and 1-year survival rate in this study were 9.2 months and 33.3%, respectively. The results were much better than in a recent phase II study that evaluated the activity of topotecan against recurrent SCLC.[17] In addition, the results of our study were comparable with those of a phase II study that evaluated the activity of amrubicin against refractory or recurring SCLC.[11] In conclusion, amrubicin is an active agent for previously treated SCLC, and the recommended doses in second-line and third-line settings are 40 mg/m^2 on days 1 to 3 and 35 mg/m^2 on days 1 to 3, respectively. Because of the greater activity of the single-agent amrubicin, further studies on amrubicin either as a single agent or in combination with other agents, such as cytotoxic or target-based agents, are warranted in previously treated SCLC patients.

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