experienced disease relapse outside the thorax and within the thorax, respectively. Two patients experienced disease relapse both within and outside the thorax. The most common first failure organ was the brain (five patients, 42%).

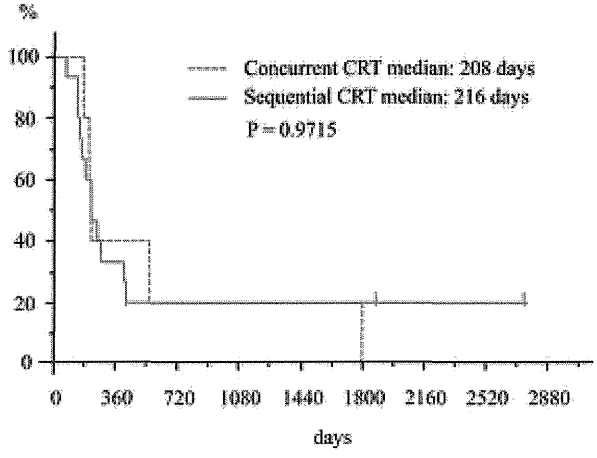

Figure 1. Kaplan–Meier curves for the progression-free survival (PFS) of patients aged 75 years or older treated with concurrent chemoradiotherapy (CRT) and sequential CRT are shown (concurrent CRT, red dashed line; sequential CRT, blue continuous line). The median PFS was 208 days in concurrent CRT and 216 days in sequential CRT. There was no statistically significant difference between the two groups (log-rank $P = 0.9715$).

Table 5 shows the adverse events in these 12 patients. Although there were moderate levels of hematological toxicities, gastrointestinal toxicities tended to be mild. It is noteworthy that Grade 3 or more severe pneumonitis occurred in four patients (33%).

DISCUSSION

Our investigation is important as it includes a considerable number of LD-SCLC patients aged 75 years or older who have been treated with CRT. Moreover, as this study documents a precise clinical course (i.e. treatment response, PFS, OS, treatment compliance and adverse events), it will enable physicians to determine the optimal treatment strategy for this category of patients.

Two previous research papers have detailed clinical course data in studies similar to ours. In one study, seven LD-SCLC patients aged 75 years or older were treated with etoposide plus cisplatin or carboplatin and with concurrent TRT (14). TRT treatment was delayed for more than 7 days in three of the seven patients. Three experienced Grade 3 or more severe febrile neutropenia, and three experienced

Table 4. Adverse events in patients treated with concurrent CRT and sequential CRT

	Concurrent chemoradiotherapy ($n = 5$)						Sequential chemoradiotherapy ($n = 15$)					
	Gr 1	Gr 2	Gr 3	Gr 4	≥Gr 3 (%)	All (%)	Gr 1	Gr2	Gr 3	Gr 4	≥Gr 3 (%)	All (%)
Leukopenia	0	0	3	2	100	100	1	6	8	0	53	100
Neutropenia	0	0	0	5	100	100	1	0	3	11	93	100
Anemia	0	4	1	0	20	100	3	7	2	0	13	80
Thrombocytopenia	2	2	1	0	20	100	6	3	3	1	27	87
Fatigue	1	1	1	0	20	60	7	2	0	0	0	60
Anorexia	2	1	1	0	20	80	6	5	0	0	0	73
Constipation	2	2	0	0	0	80	12	1	0	0	0	87
Nausea	2	2	0	0	0	80	6	1	0	0	0	47
Infection	0	2	0	0	0	40	1	1	1	0	7	20
Febrile neutropenia	0	0	3	0	60	60	0	0	2	0	13	13
Bilirubin	1	0	0	0	0	20	2	1	0	0	0	20
AST	0	0	0	0	0	0	2	0	0	0	0	13
ALT	1	0	0	0	0	20	3	0	0	0	0	20
Hyponatremia	2	0	0	1	20	60	4	0	1	1	13	40
Creatinine elevation	1	0	0	0	0	20	3	2	0	0	0	33
Pneumonitis	4	0	0	0	0	80	7	0	3	1	27	73
Esophagitis	1	3	1	0	20	100	5	4	0	0	0	60
Dermatitis	4	0	0	0	0	80	9	0	0	0	0	60
Eruption	2	0	0	0	0	40	1	1	0	0	0	13

Gr, grade; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

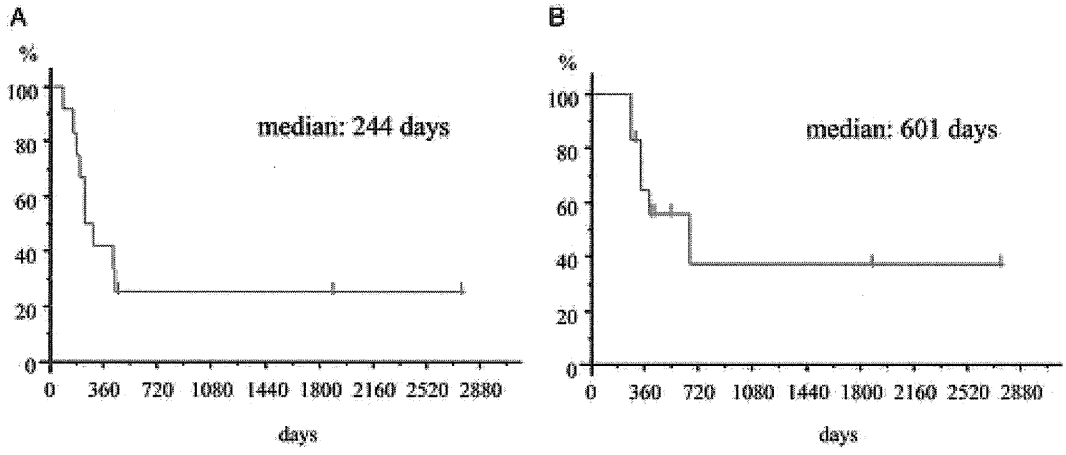

Figure 2. The Kaplan–Meier curve for the PFS (A) and overall survival (OS) (B) of 12 patients aged 75 years or older, treated with etoposide plus carboplatin followed by sequential thoracic radiotherapy is shown. The median PFS and OS were 244 and 601 days, respectively.

Table 5. Adverse events in patients treated by etoposide plus carboplatin and sequential radiotherapy, $n = 12$

	Gr 1	Gr 2	Gr 3	Gr 4	≥Gr 3 (%)	All (%)
Leukopenia	0	6	6	0	50	100
Neutropenia	0	0	3	9	100	100
Anemia	2	5	2	0	17	75
Thrombocytopenia	4	3	2	1	25	83
Fatigue	5	2	0	0	0	58
Anorexia	5	4	0	0	0	75
Constipation	9	1	0	0	0	83
Nausea	5	0	0	0	0	42
Infection	1	1	1	0	8	25
Febrile neutropenia	0	0	2	0	17	17
Bilirubin	1	1	0	0	0	17
AST	1	0	0	0	0	8
ALT	3	0	0	0	0	25
Hyponatremia	3	0	0	0	0	25
Creatinine elevation	2	2	0	0	0	33
Pneumonitis	5	0	3	1	33	75
Esophagitis	5	3	0	0	0	67

Grade 4 thrombocytopenia. One patient died due to radiation pneumonitis and this was judged as treatment-related death. In the second study, the outcome of elderly patients aged 70 years or older, five of whom were 75 years or older, who received early concurrent CRT with four cycles of etoposide plus cisplatin, was reported (15). Of the 12 patients in this report, 8 (67%) experienced Grade 3 or more severe febrile neutropenia. Of the five patients aged 75 years or older, three could not complete the four cycles of chemotherapy and all five experienced delayed TRT for more than 7 days. In our study, five patients received concurrent CRT and two could not complete the chemotherapy course due to toxicities. TRT was discontinued in one patient and another experienced delayed TRT for more than 7 days due to toxicities. These patients suffered from prolonged toxicities and their quality of life decreased for a long time. Moreover, it is speculated that fitter patients were treated by concurrent CRT and more fragile patients were treated by sequential CRT. Therefore, it is suggested that concurrent CRT is not feasible for all LD-SCLC patients aged 75 years or older. Moreover, a high frequency of discontinuation, dose reduction and omission of chemotherapy/TRT in concurrent CRT may lead to a similar PFS as that achieved with sequential CRT.

Based on the previous Phase III study which investigated chemotherapeutic regimen for elderly or poor-risk patients with ED (extensive disease)-SCLC (16) and the convenient administration schedule of carboplatin, etoposide (80 mg/m^2) on days 1–3 plus carboplatin (AUC 5) on day 1 followed by sequential TRT 45Gy in twice-daily fractions or 50 Gy in a once-daily fraction was the most frequently used treatment method for LD-SCLC patients aged 75 years or older in our institute. In our study, the major adverse events of etoposide plus carboplatin followed by sequential TRT were hematological toxicities, including neutropenia and thrombocytopenia. Gastrointestinal toxicities such as anorexia, nausea, vomiting and constipation were very mild. All of the toxicities were manageable and no treatment-related death occurred. The response rate, OS and PFS were satisfactory, when taking the patients' characteristics in our study and the results of the previous Phase II studies that evaluated CRT for LD-SCLC patients aged 70 years or older, into account (17, 18). However, as Grade 3 or more severe pneumonitis occurred in 4 of 12 patients (33%) similar to a retrospective subset analysis of LD-SCLC patients treated with etoposide plus cisplatin and concurrent early CRT in a Phase III trial (10), attention should be paid to the occurrence of radiation

pneumonitis. It may be appropriate to set the radiation field based on the tumor volume after induction chemotherapy to reduce the frequency and severity of radiation pneumonitis (19). On the other hand, the previous Phase III study have also shown etoposide plus split doses of cisplatin seems to be another standard chemotherapeutic regimen for elderly or poor-risk patients with ED-SCLC (16). Etoposide plus split doses of cisplatin on days 1–3 followed by sequential TRT could be a candidate for the standard treatment of LD-SCLC patients aged 75 years or older. However, because only three patients were treated by etoposide plus split doses of cisplatin on days 1–3 followed by sequential TRT, it is hard to lead a definitive conclusion in this study.

Our study has a few limitations. The intervals between evaluations for lesions in this study were not as accurate as those in a prospective study. The severity of non-hematological toxicities, in particular, may have been underestimated in the present study due to its retrospective nature. Patients were treated as inpatients during most of the treatment period, and the toxicity data were recorded in detail in the patients' medical records. The sample size in this study is not very large; therefore, it is difficult to reach a definitive conclusion. However, as it is not easy to collect data on a large number of LD-SCLC patients aged 75 years or older who have received CRT, this study may be useful for physicians trying to determine the optimal treatment strategy for LD-SCLC patients aged 75 years or older.

In conclusion, it is suggested that concurrent CRT is not feasible for all LD-SCLC patients aged 75 years or older. Etoposide (80 mg/m^2) on days 1–3 plus carboplatin (AUC 5) on day 1 followed by sequential TRT is one of the candidates for the standard treatment of these elderly LD-SCLC patients. A further prospective clinical trial is warranted to develop and evaluate the optimal treatment method for LD-SCLC patients aged 75 years or older.

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Conflict of interest statement

None declared.

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The Impact of Clinical Outcomes According to *EGFR* Mutation Status in Patients with Locally Advanced Lung Adenocarcinoma Who Recieved Concurrent Chemoradiotherapy

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Objectives: Among patients with locally advanced lung adenocarcinoma, the frequency of epidermal growth factor receptor (*EGFR*) and *KRAS* mutations was unknown. In addition, it has not been fully evaluated about the role of these mutations treated with concurrent chemoradiotherapy (CCR).

Methods: The clinical records of locally advanced lung adenocarcinoma patients treated with CCR at Shizuoka Cancer Center between September 2002 and December 2009 were reviewed.

Results: Forty-four patients were eligible for this study. *EGFR* mutation was detected in 13 (29.5%) of 44 patients, and *KRAS* mutation was detected in 2 (6.5%) of 31 patients. Among *EGFR* mutation status known patients, overall response rate, median progression-free survival (PFS), and median survival time were 52.3%, 11.5 months, and 35.8 months, respectively. Overall response rate was significantly higher in *EGFR* mutant group than in *EGFR* wild-type group (76.9% vs. 41.9%, $P=0.02$), but this difference did not translate into a significant PFS benefit (9.6 vs. 13.2 mo, $P=0.78$). Locoregional relapse occured less frequently in patients with *EGFR* mutation than those with *EGFR* wild-type, but not significant (15.4% vs. 32.3%, $P=0.46$). Brain was the most frequent metastatic site of relapse in *EGFR* mutant group.

Conclusions: Among locally advanced lung adenocarcinoma, *EGFR* mutation was detected in 29.5% and *KRAS* mutation was detected in 6.5%. We were not able to detect a difference in PFS or overall survival between *EGFR* mutant and wild-type patients treated with conventional CCR. Locoregional relapse was approximately half in the *EGFR* mutant group compared with the *EGFR* wild-type group; however, this finding did not reach statistical significance.

Key Words: non–small cell lung cancer (NSCLC), locally advanced, chemoradiotherapy, epidermal growth factor receptor (*EGFR*) mutation

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Lung cancer is the leading cause of cancer-related death in the world. Approximately 30% of patients with non–small cell lung cancer (NSCLC) have an unresectable locally advanced disease.[1,2] Although concurrent chemoradiotherapy (CCR) is the standard treatment in patients with unresectable, locally advanced NSCLC (LA-NSCLC), its outcome was not satisfactory. Recent randomized phase III trials have documented that the CCR with third generation regimens was effective for the treatment of unresectable LA-NSCLC as compared with that with second generation regimens, demonstrating no statistically significant difference in the overall survival (OS).[3,4] However, there have been still no established regimens for the treatment of CCR, therefore, further study is warranted.

In East Asians, epidermal growth factor receptor (*EGFR*) and *KRAS* mutations were detected in 30% and 10% of lung adenocarcinoma, respectively.[5] *EGFR* mutation is a powerful predictive marker for advanced NSCLC treated with *EGFR*-tyrosine kinase inhibitor (TKI),[6–8] whereas *KRAS* mutation is a negative predictive marker and they are mutually exclusive.[9] Recently, very favorable outcomes in 2 phase III studies of gefitinib as first-line therapy compared with platinum-based chemotherapy have been described in patients with advanced NSCLC harboring *EGFR* mutations.[8,9] The patients treated with gefitinib had promising outcomes, median survival time of 30.5 months, and 2-year survival rate of 61.4%. Therefore, selected NSCLC patients, most of them adenocarcinoma, may survive >2 years. Recent in vitro study demonstrated the radiosensitivity in NSCLC cell lines harboring *EGFR* mutation.[10] Clinically, it remains unknown whether CCR is effective for the treatment of locally advanced lung adenocarcinoma with *EGFR* mutation as compared with that with *EGFR* wild-type. There is still no data about the frequency of *EGFR* mutation and the outcome after CCR in patients with locally advanced lung adenocarcinoma. Therefore, we conducted a retrospective study to examine the clinical outcome after CCR according to *EGFR* mutation status in locally advanced lung adenocarcinoma.

MATERIALS AND METHODS

Patient Selection

Between September 2002 and December 2009, we reviewed the clinical records of 90 consecutive, unresectable, locally advanced lung adenocarcinoma patients treated with

CCR at Shizuoka Cancer Center. The eligibility criteria of this study was as follows: (1) histologically or cytologically proven adenocarcinoma; (2) chemoradiotherapy naive, with measurable target legion on physical examination, chest x-ray, and computed tomography (CT) of the chest; (3) Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 0 to 1; (4) received curable thoracic radiotherapy over 50 Gy; and (5) with adequate specimens for *EGFR* mutation analysis. Of 90 patients who received CCR, 44 were eligible for this study. Most common reason for exclusion was lack of adequate samples to analyze *EGFR* mutation (45 patients). One patient was excluded due to his general condition (ECOG PS of 2).

EGFR and *KRAS* Mutations Analysis

EGFR mutation analysis of cytologic or histologic specimens was screened by the PNA-LNA PCR clamp method (untill March 2010) or Cycleave method (between April and December 2010) as previously described.[11,12] *KRAS* mutation analysis of histologic specimens was screened by pyrosequencing method as previously described.[13]

Treatment Methods

Treatment was composed of CCR and subsequent consolidation chemotherapy. Chemotherapy regimen was selected at investigator's discretion. All patients were treated with a linear accelerator photon beam of 4 MV or more. The primary tumor and involved nodal disease was planned to receive at least 60 Gy in 2 Gy fractions over 6 weeks. Our radiation technique is based on elective nodal irradiation. The radiation fields contain the primary tumor, ipsilateral hilum, and mediastinal nodal areas from the paratracheal to subcarinal lymph nodes. The contralateral hilum was not included, and the supraclavicular areas were not to be treated routinely.

Evaluation of Response and Statistical Analysis

Tumor response was classified in accordance with the Response Evaluation Criteria for Solid Tumors (RECIST), version 1.0. In almost all patients, tumor response was assessed for every 2 courses of chemotherapy. After the treatment period, chest CT was performed every 2 to 3 months during the first year and at 3- to 6-month intervals thereafter. Positron emission tomography (PET) or PET-CT using 2-[^{18}F]-fluoro-2-deoxy-D-glucose was favorable at 6 to 12-month intervals if available. Magnetic resonance imaging of the brain was performed only when clinical signs and symptoms suspicious for brain involvement were present. Progression-free survival (PFS) was assessed from the first day of treatment with CCR to the earliest signs of disease progression as determined by CT or magnetic resonance imaging using RECIST criteria, or death from any cause. Probability values of <0.05 indicated a statistically significant difference. Differences between covariates in patients with *EGFR* mutation and wild-type were analyzed using the Fisher exact tests and χ^2 tests. The Kaplan-Meier method was used to estimate survival as a function of time, and survival difference were analyzed by the log-rank test. All the analyses were performed using JMP version 7 (SAS Institute Inc.).

RESULTS

Patient Characteristics

Patient's characteristics are listed in Table 1. The median follow-up time for the 19 censored patients was 37.7 months (range, 8.3 to 75.6 mo). Fifty-seven percent of patients had died and 84% of patients had disease progression at the time of this analysis. Forty-four samples were available for *EGFR* mutation analysis and 31 were available for *KRAS* mutation analysis.

TABLE 1. Patient's Characteristics

Characteristics	*EGFR* Mutation [N (%)] Mutant (N=13)	Wild-type (N=31)	*P*
Age (y)			0.05
Median	68	64	
Range	55-80	40-76	
Sex [n (%)]			0.29
Male	8 (61.5)	24 (77.4)	
Female	5 (38.5)	7 (22.6)	
Smoking status			<0.05
Non or light smoker	6 (46.2)	5 (16.1)	
Heavy smoker	7 (53.8)	24 (77.4)	
Unknown	0	2 (6.5)	
ECOG performance status [n (%)]			0.68
0	10 (76.9)	22 (71.0)	
1	3 (23.1)	9 (29.0)	
Clinical stage [n (%)]			0.32
IIIA	9 (69.2)	15 (48.4)	
IIIB	4 (30.8)	16 (51.6)	
Chemotherapy regimen [n (%)]			0.57
Platinum-based regimen	11 (84.6)	29 (93.5)	
Monotherapy	2 (15.4)	2 (6.5)	
RT dose (Gy)			0.76
Median	60	60	
Range	60-74	56-74	

ECOG indicates Eastern Cooperative Oncology Group; *EGFR*, epidermal growth factor receptor; RT, radiation therapy.

Among 44 patients, 32 were male and 12 were female. The median age was 65 years (range, 40 to 80 y). Thirty-two (72.8%) patients were with ECOG PS of 0, and 24 (54.5%) patients were clinical stage IIIA. Forty (90.9%) patients received platinum-based regimen and the other patients received monotherapy. Fifteen patients were treated with cisplatin plus S-1, 13 patients with cisplatin plus vinorelbine, and 9 patients with carboplatin plus paclitaxel. The median radiation doses were 60 Gy (range, 56 to 74 Gy).

EGFR mutation was detected in 13 patients (29.5%). No statistically significant difference in the age, sex, ECOG PS, and disease stage was observed between the groups. Only smoking status yielded a statistically significant difference ($P<0.05$). Most frequent type of mutation was L858R in exon 21 (9 patients). Deletion in exon 19 was observed in 2 patients and the rest site of mutation was detected in exon 18. T790M mutation in exon 20 was not detected. *KRAS* mutation was detected in 2 patients (6.5%). Both of them are codon 12 mutations. *EGFR* and *KRAS* mutations were mutually exclusive.

Efficacy and Survival Analysis

Among *EGFR* status known patients (n=44), overall response rate (ORR), median PFS, and median OS were 52.3%, 10.8 months, and 30.9 months, respectively. The ORR was significantly higher in *EGFR* mutant group than *EGFR* wild-type group (76.9% vs. 41.9%, $P=0.02$). However, this difference did not translate into a significant PFS benefit (9.6 vs. 13.2 mo, $P=0.78$, Fig. 1). The median OS seemed to be longer in *EGFR* mutant group than *EGFR* wild-type group

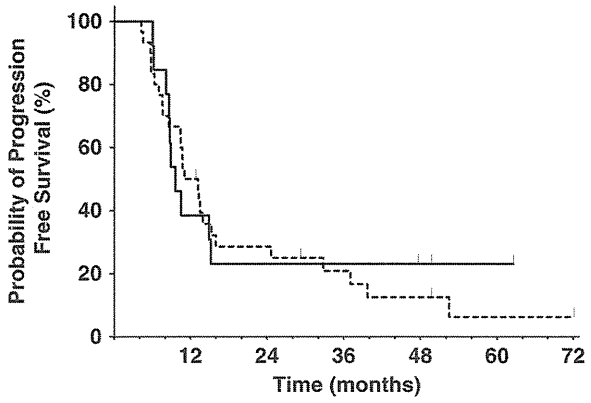

FIGURE 1. Kaplan-Meier–estimated PFS curve in patients with *EGFR* mutation (bold line, 9.6 mo) and *EGFR* wild-type (dash line, 13.2 mo). Significant difference in the PFS was not observed by *EGFR* mutation status; hazard ratio 0.90; 95% confidence interval, 0.41-1.82; $P=0.78$. *EGFR* indicates epidermal growth factor receptor; PFS, progression-free survival.

(57.9 vs. 30.7 mo, Fig. 2). Relapse pattern after initial treatment is listed in Table 2. Thirty-six (81.8%) patients demonstrated disease progression during the follow-up period (median, 28.8 mo). Twenty-four patients developed distant metastasis, 9 patients locoregional relapse, and 3 patients developed both. Locoregional relapse was observed only in 2 patients (15.4%) in *EGFR* mutant group, whereas 10 patients (32.3%) demonstrated locoregional relapse in *EGFR* wild-type group. Brain was the most frequent metastatic site (6 patients) in *EGFR* mutant group. Salvage therapy was also reviewed. Twenty-seven (75.0%) of 36 relapsed patients received second-line chemotherapy. All relapsed patients with *EGFR* mutation were treated with *EGFR*-TKI at any subsequent lines.

DISCUSSION

This is a retrospective study to evaluate the clinical significance of *EGFR* mutation in patients with unresectable, locally advanced lung adenocarcinoma who received CCR. *EGFR* and *KRAS* mutations were detected in 29.5% and 6.5%,

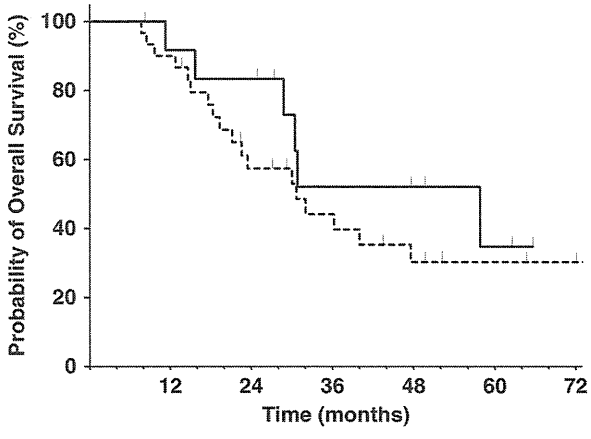

FIGURE 2. Kaplan-Meier–estimated overall survival curve in patients with *EGFR* mutation (bold line, 57.9 mo) and *EGFR* wild-type (dash line, 30.7 mo). *EGFR* indicates epidermal growth factor receptor.

TABLE 2. Relapse Pattern After Chemoradiotherapy in Each Group

Variables	*EGFR* Mutation [N (%)] Mutant (N=13)	Wild-type (N=31)	*P*
Overall recurrence	10 (76.9)	26 (83.9)	0.68
Locoregional only	1 (7.7)	8 (25.8)	0.24
Locoregional + distant	1 (7.7)	2 (6.5)	1.00
Distant only	8 (61.5)	16 (51.6)	0.55
Brain	6 (46.2)	4 (12.9)	0.04
Pulmonary metastasis	2 (15.4)	2 (6.5)	0.57

EGFR indicates epidermal growth factor receptor.

respectively, and they are mutually exclusive. ORR was significantly higher in *EGFR* mutant group than *EGFR* wild-type group, but this difference did not translate into a significant PFS benefit. Locoregional relapse occurred less frequently in patients with *EGFR* mutation than those with *EGFR* wild-type, but not significant.

There were few reports described about the frequency of *EGFR* mutation among stage III LA-NSCLC. Kosaka et al[14] reported that the prevalence of *EGFR* mutation was not different according to disease staging (stage I vs. stage II to IV, $P=0.34$). Mak et al[15] reported that *EGFR* mutation was detected in 24.8% of NSCLC patients treated by curative thoracic radiation. Recent phase II trial for LA-NSCLC has documented that *EGFR* mutation was detected in 28.9% of NSCLC patients.[16] This is corresponding to the result of our study. As the frequency of *EGFR* mutation is detected in approximately 30% of stage IIIB/IV adenocarcinoma, no significant difference in the positive rate of *EGFR* mutation seems to be recognized between stage III and metastatic adenocarcinoma patients. Only 2 patients were found to have *KRAS* mutation in our analysis. Because of this limited sample numbers, it was difficult to evaluate the patient's demographics and prognosis according to *KRAS* mutation in the present study.

Our survival analysis demonstrated that the median PFS was not significantly different between patients with or without *EGFR* mutation. The median OS seemed to be longer in *EGFR* mutant group than *EGFR* wild-type group, because all relapsed patients with *EGFR* mutation were treated with *EGFR*-TKI at any subsequent lines. In the pivotal phase III trials for metastatic NSCLC patients with *EGFR* mutation, *EGFR*-TKI demonstrated higher ORR and longer PFS than platinum doublets.[8,9] However, we should keep in mind that patients were never cured once they relapsed. Okamoto et al[17] reported a feasibility study of gefitinib and thoracic radiation therapy for stage III LA-NSCLC. As 5 of 9 patients did not complete the planned treatment due to disease progression or pneumonitis, they concluded gefitinib and thoracic radiation therapy was not feasible for unselected population. However, 2 patients with *EGFR* mutation completed treatment without interruption. Both of them lived for >5 years and their initial site of relapse was brain. Niho et al[18] conducted another feasibility study of gefitinib and concurrent thoracic radiotherapy for unresectable LA-NSCLC. As patients were highly selected, most of them were Japanese, had adenocarcinoma, and never or light smokers. In this trial, the toxicity was acceptable, taking into account the incidents of pneumonitis (2 of 38 patients). Thus, further study is warranted for evaluating the

therapeutic possibility of CCR including gefitinib or erlotinib according to *EGFR* mutation status in stage III LA-NSCLC.

In the phase III trial, locoregional relapse was observed in approximately 40% of LA-NSCLC patients treated with CCR.[4] Recently, Mak et al[15] described that *EGFR* mutation was an independent factor of locoregional relapse (hazard ratio = 0.45) among LA-NSCLC. However, their analysis contained various types of treatment modalities such as curative radiation only or induction CCR followed by surgery. In the present study, locoregional relapse was observed in 15.4% among *EGFR* mutant group, whereas 32.3% among *EGFR* wild-type group. Although we could not demonstrate statistically significant difference, this finding was concordant with the study by Mak and colleagues, and supports the preclinical data that *EGFR* mutant cells were radiosensitive. Further investigation is warranted for confirming these results.

The present study has several limitations. Our population was small sample size. This may bias the comparison of outcomes after CCR between patients with *EGFR* mutation and *EGFR* wild-type. Because of the low accrual rate of available specimens, 44 of 90 (47.8%) adenocarcinoma patients were screened for the analysis of *EGFR* mutation. Therefore, 46 patients had an unknown status of *EGFR* mutation.

In conclusion, *EGFR* mutation was detected in 29.5% and *KRAS* mutation was detected in 6.5% among locally advanced lung adenocarcinoma. *EGFR* mutation did not predict PFS after CCR but it could predict locoregional control. Our preliminary study suggests that conventional CCR may not be the most recommended treatment for stage III LA-NSCLC patients with *EGFR* mutation. Further studies may be considered for evaluating the therapeutic possibility of CCR adding *EGFR*-TKI in this population.

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Large Cell Neuroendocrine Carcinoma of the Lung: Is it Possible to Diagnose from Biopsy Specimens?

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Objective: We have recently proposed new diagnostic criteria for high-grade non-small cell neuroendocrine carcinoma, i.e. possible large cell neuroendocrine carcinoma, in biopsy specimens and have started a clinicopathological comparative study of high-grade neuroendocrine carcinomas in an advanced stage. This study aimed to elucidate the usefulness of our diagnostic criteria for inoperable advanced large cell neuroendocrine carcinoma and to know the true incidence of large cell neuroendocrine carcinoma among lung cancers.
Methods: We reviewed all cancer lesions (1040 specimens) obtained by transbronchial lung biopsies in our hospital from 2002 to 2009 and selected 38 biopsy specimens that satisfied our diagnostic criteria for high-grade non-small cell neuroendocrine carcinoma. All 38 cases were clinicopathologically investigated and all biopsy specimens were precisely studied for their morphological characteristics.
Results: Clinicopathological information about the selected 38 cases was very similar to the clinicopathological characteristics of large cell neuroendocrine carcinoma reported. Of 38 cases, six were at Stage I, II or IIIA, underwent surgery, and the diagnosis was confirmed to be large cell neuroendocrine carcinoma using surgical tumor specimens. In the 38 biopsy specimens, features of neuroendocrine morphology such as organoid nesting, peripheral palisading and rosette formation were not frequent histological features and the majority of tumor cells contained nuclei with a fine chromatin pattern. Mitoses were difficult to find; however, immunohistochemical Ki-67/MIB1 labeling indices were quite useful for evaluating proliferative activity, which ranged from 43.4 to 99.0%.
Conclusions: Our study showed the diagnostic potential of using biopsy specimens for large cell neuroendocrine carcinoma, and we herein proposed more simplified diagnostic criteria for possible large cell neuroendocrine carcinoma in practical diagnostic use.

Key words: large cell neuroendocrine carcinoma – biopsy diagnosis – Ki-67 – neuroendocrine markers – small cell carcinoma

INTRODUCTION

Lung carcinoma is clinically classified into two categories, i.e. small cell lung carcinoma (SCLC) and non-SCLC (NSCLC), with regard to their response to chemoradiotherapy. In 1991, Travis et al. (1) separated a group of high-grade neuroendocrine carcinoma from NSCLC and proposed the new histological category of large cell neuroendocrine carcinoma (LCNEC), which was adopted into the WHO classification in 1999 (2) and retained in the 2004 WHO classification (3). The main criteria for diagnosing LCNEC are: (1) large

cell morphology; (2) high mitotic rate of more than 11 or greater/10 high power fields (HPFs); and (3) the detection of neuroendocrine morphology and immunohistochemical markers (1–3). However, it became clear that the clinical features of LCNEC are similar to those of SCLC, and the differential diagnosis between LCNEC and SCLC is quite difficult in some cases (4,5). The morphological and clinical similarities of these high-grade neuroendocrine carcinomas are problematic for clinicians and pathologists (1,4,5).

Although the role of chemotherapy in LCNEC is uncertain, Iyoda et al. (6) have reported that patients who received adjuvant chemotherapy had a better prognosis than patients who did not receive adjuvant chemotherapy. Moreover, recent advances in chemotherapy have elucidated that platinum-based adjuvant chemotherapy is effective and significantly improves the survival of patients with LCNEC compared with non-platinum-based adjuvant chemotherapy, suggesting that the initial treatment response of LCNEC to chemotherapy might be similar to that of SCLC (7–10). The results of chemotherapy in previous LCNEC studies were obtained by adjuvant chemotherapy after surgery and the diagnosis of LCNEC was made by using resected surgical materials. Not only for resectable LCNEC, but also for non-resectable LCNEC the pathological diagnosis should be made for selecting an appropriate chemotherapy. However, for the diagnosis of LCNEC in a biopsy specimen, the criteria of LCNEC cannot fit because, for example, the mitotic rate cannot necessarily be counted in 10 HPFs in a small necrosis-rich and/or crushed specimen. Thus, for the tumors that can possibly be LCNEC, another criterion for the diagnosis of possible LCNEC is required. Therefore, we focused attention on unresectable high-grade neuroendocrine carcinoma of the lung and aimed to perform a retrospective comparative study in order to know the chemotherapy response of LCNEC and SCLC (9). This comparative study used biopsy specimens instead of surgical specimens to diagnose LCNEC correctly and to differentiate LCNEC from SCLC. For this purpose, we modified the diagnostic criteria of LCNEC proposed by Travis et al. (1) and proposed new diagnostic criteria of high-grade non-small cell neuroendocrine carcinoma (HNSCNEC), which likely includes most LCNEC (9).

Using our criteria of HNSCNEC, at least two papers have been published so far and it was concluded that the efficacy of chemotherapy for unresectable LCNEC is comparable with that of SCLC (9,10). In this study, we morphologically reviewed all lung biopsy specimens obtained from our hospital from 2002 to 2009 and aimed to improve the criteria of HNSCNEC for better practice with using biopsy specimens and to estimate the true frequency of LCNEC in lung cancer.

PATIENTS AND METHODS

Biopsy Samples

From September 2002 to December 2009, transbronchial lung biopsy was performed on 1566 patients with lung tumor at Shizuoka Cancer Center Hospital. Using hematoxylin and eosin (H&E)-stained paraffin sections of these biopsy specimens, we histologically reviewed their diagnosis. Of 1566 biopsy cases, 1040 were evaluated to have carcinoma tissue, with a diagnosis of adenocarcinoma (518 cases), squamous cell carcinoma (318 cases), adenosquamous carcinoma (6 cases), SCLC (121 cases), large cell carcinoma (24 cases) and pleomorphic carcinoma (15 cases). Finally, we selected 38 HNSCNECs according to our criteria (9). Selected from the criteria shown in Table 1 (9), we used the following as essential conditions: no differentiation to squamous cell carcinoma or adenocarcinoma, positive immunostaining for at least one of neuroendocrine markers, large nuclear size with moderate or marked nuclear atypia, a Ki-67/MIB1 labeling index higher than 40%, and nuclear features (fine chromatin and/or prominent nucleoli) or one of the features of neuroendocrine morphology such as organoid nesting, peripheral palisading, rosettes and/or trabecular architecture.

We excluded poorly differentiated adenocarcinomas by positive periodic acid-schiff-alcian blue mucin staining after diastase digestion. We also excluded squamous cell carcinomas by p63 and keratin 5/6 immunopositivity. The immunohistochemical Ki-67 labeling index was the indicator of high-grade malignancy. Neuroendocrine carcinoma was defined by at least one positive neuroendocrine marker of chromogranin A, synaptophysin and neural cell adhesion molecule (NCAM) and by the neuroendocrine morphology.

Clinical information of these 38 patients was obtained from patients' records (Table 2).

Table 1. Applied criteria of high-grade non-small cell neuroendocrine carcinoma (HNSCNEC)

1. Solid tumor nesting without either acinar or squamous differentiation
2. Moderate or marked cellular atypia
3. Large cell size with low nuclear/cytoplasmic ratio or abundant cytoplasm
4. Vesicular and/or fine nuclear chromatin
5. Frequent nucleoli
6. Positive immunostaining for one or more neuroendocrine markers (NCAM, chromogranin A and synaptophysin)
7. Ki-67/MIB1 labeling index >40%
8. Frequent mitosis
9. Frequent massive necrosis
10. Intercellular space (cleft) with loose intercellular adhesion
11. Organoid nesting, basal palisading, rosettes and/or trabecular architecture

Proposed criteria for diagnosis of HNSCNEC using biopsy specimens (Table 2 of ref. (9)).

Immunohistochemistry

For immunostaining, 3 μm-thick sections were prepared from the formalin-fixed and paraffin-embedded tumor specimens. After deparaffinization and blocking of endogenous

Table 2. Clinical features of 38 biopsy cases used in this study

Case number	Age	Gender	Smoking	Tumor size on CT (cm)	Tumor margin on CT	Tumor location	Site of biopsy	T	N	M	Stage	Initial pathological diagnosis	Surgery	Status of patients (months)
1	74	Male	Yes	3.6 × 2.0	Non-lob	p	rt M	1	0	0	IA	LCNEC	Yes	NED (73)
2	70	Male	Yes	3.1 × 2.8	lob	p	lt U	2	0	0	IB	LCNEC	Yes	DOD (68)
3	63	Male	Yes	4.0 × 3.0	Non-lob	p	rt M	2	0	0	IB	Poorly diff. carcinoma	Yes	NED (5)
4	82	Male	Yes	3.8 × 2.8	lob	p	lt U	2	0	0	IB	High-grade NE carcinoma	Yes	AWD (17)
5	56	Male	Yes	3.8 × 3.6	lob	p	lt U	2	1	0	IIB	High-grade NE carcinoma	Yes	NED (14)
6	64	Male	Yes	2.4 × 1.9	lob	p	lt L	3	2	0	IIIA	High-grade NE carcinoma	Yes	NED (8)
7	57	Male	Yes	3.5 × 3.5	Non-lob	c	rt U	4	3	0	IIIB	Adenocarcinoma	No	NED (61)
8	72	Male	Yes	2.5 × 1.8	lob	p	rt L	1	3	0	IIIB	Poorly diff. adenocarcinoma	No	DOD (9)
9	67	Male	Yes	4.5 × 4.5	Non-lob	p	lt L	4	3	1	IV	Combined SCLC	No	DOD (8)
10[a]	71	Male	Yes	7 × 6 × 4.5	lob	p	lt L	2	1	1	IV	Non-small cell NE carcinoma	No	DOD (11)
11[a]	74	Male	Yes	3.9 × 2.7	Non-lob	c	rt U	2	3	1	IV	High-grade NE carcinoma	No	DOD (4)
12	75	Male	Yes	2.0 × 1.8	Non-lob	p	lt U	1	2	1	IV	LCNEC	No	NED (62)
13[a]	75	Male	Yes	4.5 × 3.6	lob	c	lt U	2	3	1	IV	LCNEC	No	DOD (3)
14	66	Male	Yes	4.8 × 2.6	Non-lob	p	lt L	2	2	1	IV	High-grade NE carcinoma	No	DOD (7)
15[a]	63	Male	Yes	2.5 × 2.1	Non-lob	p	rt U	1	3	1	IV	SCLC	No	DOD (17)
16	84	Male	Yes	8.7 × 5.0	lob	p	rt L	2	1	1	IV	LCNEC	No	LTF (3)
17	67	Male	Yes	2.6	Non-lob	p	rt U	4	0	1	IV	LCNEC	No	DOD (2)
18[a]	74	Male	Yes	6.5 × 6.0	lob	p	rt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (9)
19	74	Male	Yes	7.5 × 6.0	lob	p	rt U	2	2	1	IV	LCNEC	No	DOD (3)
20	62	Female	Yes	5.3 × 4.3	Non-lob	p	rt L	2	3	1	IV	Large cell carcinoma	No	DOD (7)
21	70	Male	Yes	7.0 × 3.5	lob	p	rt L	4	3	1	IV	Large cell carcinoma	No	DOD (7)
22	76	Male	No	3.0 × 2.3	Non-lob	c	lt L	1	3	1	IV	Combined SCLC	No	DOD (11)
23	59	Male	Yes	8.0 × 5.5	Non-lob	c	rt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (12)
24	73	Male	Yes	2.3 × 2.0	lob	p	rt U	4	0	0	IV	Poorly diff. adenocarcinoma	No	LTF (22)
25	64	Male	Yes	3.5 × 3.4	lob	c	lt L	4	3	1	IV	LCNEC	No	DOD (11)
26	77	Female	Yes	3.8 × 3.0	lob	p	lt L	3	1	1	IV	Large cell carcinoma	No	DOD (20)
27	62	Male	Yes	Unknown	Non-lob	c	rt MB	TX	NX	1	IV	High-grade NE carcinoma	No	LTF (1)
28	89	Male	Yes	4.7 × 3.7	Non-lob	p	rt M	2	3	1	IV	Large cell carcinoma	No	LTF (1)
29	67	Male	Yes	6.2 × 3.6	Non-lob	c	rt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (3)
30	81	Male	No	Unknown	Non-lob	c	lt MB	1	0	0	IV	Large cell carcinoma	No[b]	DOD (16)
31	59	Female	Yes	1.5 × 1.3	lob	c	lt U	1	3	1	IV	LCNEC	No	DOD (7)
32	63	Male	Yes	5.2 × 5.0	lob	p	rt U	4	3	1	IV	Carcinoma	No	DOD (16)
33	69	Male	No	5.5 × 3.2	lob	p	lt U	4	2	1	IV	Large cell carcinoma	No	AWD (31)
34	65	Male	Yes	4.7 × 4.1	lob	p	lt U	4	1	1	IV	LCNEC	No	LTF (17)
35	76	Male	Yes	14.4 × 8.5	Non-lob	c	lt U	4	3	1	IV	High-grade NE carcinoma	No	DOD (2)
36	64	Male	Yes	9.6 × 4.6	Non-lob	c	lt L	4	3	1	IV	Poorly diff. carcinoma	No	DOD (22)
37	65	Female	Yes	2.4 × 1.5	lob	p	lt U	1	3	1	IV	LCNEC	No	DOD (17)
38	67	Male	Yes	6.1 × 4.9	lob	p	rt L	4	3	1	IV	High-grade NE carcinoma	No	DOD (2)

CT, computed tomography; non-lob, non-lobulated margin; p, peripherally located; rt, right; M, middle lobe; LCNEC, large cell neuroendocrine carcinoma; NED, no evidence of disease; lob, lobulated margin; lt, left; U, upper lobe; DOD, dead of disease; NE, neuroendocrine; AWD, alive with disease; L, lower lobe; c, centrally located; SCLC, small cell lung cancer; LTF, lost to follow-up; MB, main bronchus; TX, primary tumor cannot be assessed by imaging; NX, regional lymph nodes cannot be assessed.

[a]The case was examined in ref. (9).

[b]Patient with a history of lung cancer resection 7 years before.

peroxidase activity by 0.3% hydrogen peroxide in methanol, antigen retrieval was carried out using the conventional autoclave method (for 10 min at 121°C) with 0.01 M sodium citrate buffer (pH 6.0), if necessary. The details of the primary antibodies were as follows: NCAM (clone NCC-Lu-243; Nippon Kayaku, Tokyo, ×200), chromogranin A (code No. A0430; DAKO, Glostrup, Denmark, ×5000), synaptophysin (Cat No. 261-01; SIGNET, Dedham, MA, USA, ×100), Ki-67/MIB1 (clone MIB1, code No. M7240; DAKO, ×100), p63 (clone 4A4, Cat No. MS-1081-P; Lab Vision, Kalamazoo, MI, USA, ×500) and keratin 5/6 (code No. M7237; DAKO, ×100). The sections were incubated with the primary antibody for 30 min at 37°C and followed by the Dako EnVision+® detection system (code K4001; Dako Cytomation North America, Inc., CA, USA) and diaminobenzidine as the chromogen to visualize the antigens according to the manufacture's instructions. Negative control slides of each case without first antibody reaction and positive control slides of other normal organ tissue for each antibody were always used and stained simultaneously.

Histological Comparison Between Biopsy and Resected Specimens of Six Resected Cases

Six cases were resected after biopsy because they were in the early stages. We compared the histological findings of these six pairs of biopsy and surgical specimens.

RESULTS

Clinicopathological Findings

Clinicopathological information about the 38 HNSCNEC cases used in this study is shown in Table 2. By computed tomography (CT) imaging of these tumors, 20 tumors (52.6%) showed solid masses with a sharply lobulated margin and 26 tumors (68.4%) were located in the periphery of the lung. The patients' average age was 69.4 (range 56–89 years). Men predominated (M:F = 34:4) and 35 out of 38 patients were smokers (92.1%). Thirty patients (78.9%) were clinically at Stage IV at diagnosis and 32 (84.2%) were unresectable. Sixteen patients (42.1%) had intrapulmonary metastasis and 23 (60.5%) had distant metastasis at the time of diagnosis, in the brain (13 patients), bone (12 patients), liver (8 patients) and adrenal gland (6 patients). Six tumors at clinical TNM Stage I, II or IIIA were surgically resected and pathologically diagnosed as LCNEC from surgical specimens.

In 38 HNSCNEC cases, serum pro-gastrin releasing peptide (pro-GRP; normal value <46.0 pg/ml), carcinoembryonic antigen (normal value <5.0 ng/ml) and neuron-specific enolase (normal value <10.0 ng/ml) levels were elevated in 13 cases (13/37, 35.1%), 24 cases (24/37, 64.9%) and 27 cases (27/38, 71.1%), respectively.

Table 3. Morphological analysis of biopsies in 38 patients, of which 6 were resected and a diagnosis of LCNEC was made

Histological findings	Number of biopsies (%)	Number of biopsies in operated cases (%)
Fine nuclear chromatin	30 (78.9)	6 (100)
Intercellular clefts	21 (55.3)	4 (66.7)
Prominent nucleoli	18 (47.4)	2 (33.3)
Massive necrosis	17 (44.7)	2 (33.3)
Organoid nesting	14 (36.8)	1 (16.7)
Peripheral palisading	6 (15.8)	1 (16.7)
Rosette formation	6 (15.8)	1 (16.7)
Trabecular arrangement	5 (13.2)	0 (0)

Histological and Immunohistochemical Analyses

Initial pathological diagnoses of 38 HNSCNEC biopsy specimens were shown in Table 2. Eleven cases were finally diagnosed as LCNEC, and non-small cell neuroendocrine carcinoma (case No. 10) was most likely to be LCNEC. Eleven cases were given the diagnosis of high-grade neuroendocrine carcinoma, which seemed to be difficult to differentiate from SCLC. On the other hand, only three cases were diagnosed as SCLC including the combined type. Eleven cases were poorly differentiated carcinoma including large cell carcinoma and adenocarcinoma. These results indicated that two-thirds of HNSCNEC biopsy cases suggested to the pathologists the diagnosis of high-grade neuroendocrine carcinoma and one-third was histologically beyond the scope of neuroendocrine carcinoma.

All HNSCNECs were composed of NSCLC with large nuclei, three times larger than resting lymphocytes and abundant cytoplasm or a low nuclear/ cytoplasmic ratio. As shown in Table 3, morphological architectural characteristics of LCNEC proposed by Travis et al. (1) were reviewed in the 38 biopsy specimens used in this study. With regard to neuroendocrine morphology, organoid nesting (Fig. 1a) was the most frequently observed structure, but its frequency was only 36.8% of all specimens. Peripheral palisading, rosette formation (Fig. 1b) and trabecular arrangement were observed in only 6 (15.8%), 6 (15.8%) and 5 (13.2%) specimens, respectively. In other morphological characteristics of LCNEC, massive necrosis (Fig. 1c) was seen in 17 biopsy specimens (44.7%). Although most tumor cells contained nuclei with a fine chromatin pattern (Fig. 1d) and faint or visible nucleoli, prominent nucleoli (Fig. 1d, arrows) were observed in about half of all specimens (47.4%). Again, in about half of all biopsy specimens (55.3%), tumor cells had a distinct cell border with intercellular clefts and were often discohesive (Fig. 1b and d).

We counted mitotic figures in all 38 tumor specimens. Among them, 11 tumors lacked enough area for mitotic counting, that is to say, 9 HPFs or less. There were 7 tumors

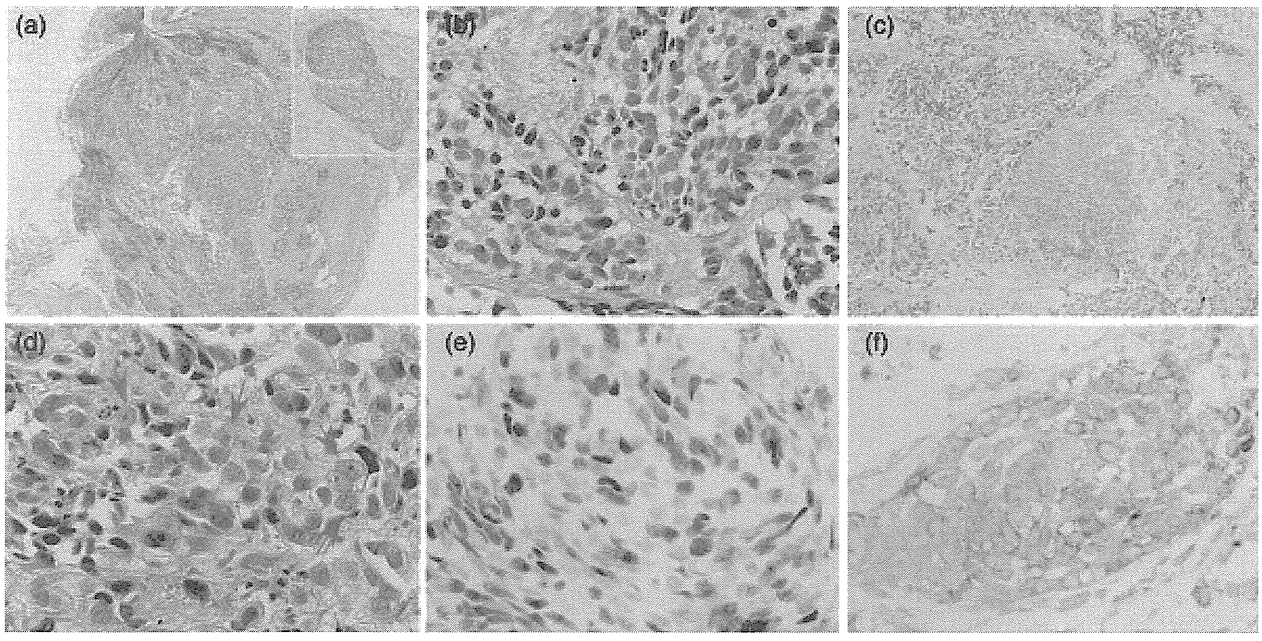

Figure 1. Characteristic morphological features of 38 biopsy specimens used in this study. In the low-powered view [a, hematoxylin and eosin (H&E), ×4], organoid nesting (a, inset) and massive necrosis (c, H&E, ×10) are recognized structure features in the biopsy specimen. In the high-powered view, tumor cells reveal special arrangements such as rosette formation (b, H&E, ×40), intercellular cleft (b) as well as nuclear characteristics such as a fine chromatin pattern (d, H&E, ×40) and prominent nucleoli (d, arrows, H&E, ×40). The immunohistochemistry shows high nuclear positivity of Ki-67 (e, ×40) and membrane positivity of the neural cell adhesion molecule (NCAM) (f, ×40) in the majority of biopsy specimens.

in which no mitotic figure was found, and another 7 tumors which had a high mitotic rate consistent with 11 or greater per 10 HPFs. In contrast to the mitotic counting, Ki-67/MIB1 labeling indices could be evaluated in all 38 tumor specimens (Fig. 1e), which ranged from 41.9 to 99.0% (median: 80.5%). The 7 biopsy specimens with 11 or more mitoses/10 HPFs showed high Ki-67/MIB1 labeling indices from 64.5 to 98.0% (median: 87.3%).

The results of the neuroendocrine immunophenotype in 38 HNSCNECs are shown in Table 4. NCAM (Fig. 1f) was the most frequently observed neuroendocrine marker (78.9%) among them. There were 12 (31.6%) triple marker-positive cases. No tumor was positive for chromogranin A alone.

As shown in Table 2, six patients underwent surgery after biopsy, and all resected tumors were pathologically examined and diagnosed as LCNEC. In case No. 1 (Table 2), for example, the biopsied specimen showed organoid nesting (Fig. 2a) and rosette formation, and tumor cells had abundant cytoplasm and crushed nuclei with a fine chromatin pattern (Fig. 2b). The immunohistochemistry elucidated NCAM (Fig. 2c) in addition to a high Ki-67/MIB1 labeling index (Fig. 2d). This biopsied tumor was pathologically diagnosed as strongly suggestive of LCNEC. As this tumor was peripherally located and clinically showed T1N0M0 (Stage IA), a right middle lobectomy was carried out after biopsy. Morphologically, the resected tumor was composed of large tumor cells with abundant cytoplasm, and showed fine or vesicular nuclei with prominent nucleoli and frequent mitoses (Fig. 3a and b). There was a necrotic area and tumor cells formed characteristic intercellular clefts and rosettes. Immunohistochemical results were almost identical to those of the biopsy specimen. Finally, the diagnosis of LCNEC was confirmed for this tumor.

Table 4. Distribution of immunohistochemical results of neuroendocrine markers in 38 biopsy specimens

Positively stained markers	Number of positive biopsy specimens (%)
NCAM	30 (78.9)
NCAM, SYN, CGA	12 (31.6)
NCAM, SYN	3 (7.9)
NCAM, CGA	1 (2.6)
NCAM alone	14 (36.8)
SYN	23 (60.5)
SYN, CGA	5 (13.2)
SYN alone	3 (7.9)
CGA	18 (47.4)
CGA alone	0 (0)

NCAM, neural cell adhesion molecule; SYN, syanptophysin; CGA, chromogranin A.

Histological details of six biopsy specimens with a confirmed diagnosis of LCNEC are shown in Tables 3 and 5. Although the sample size was small, morphological characteristics showed a similar tendency to those of other

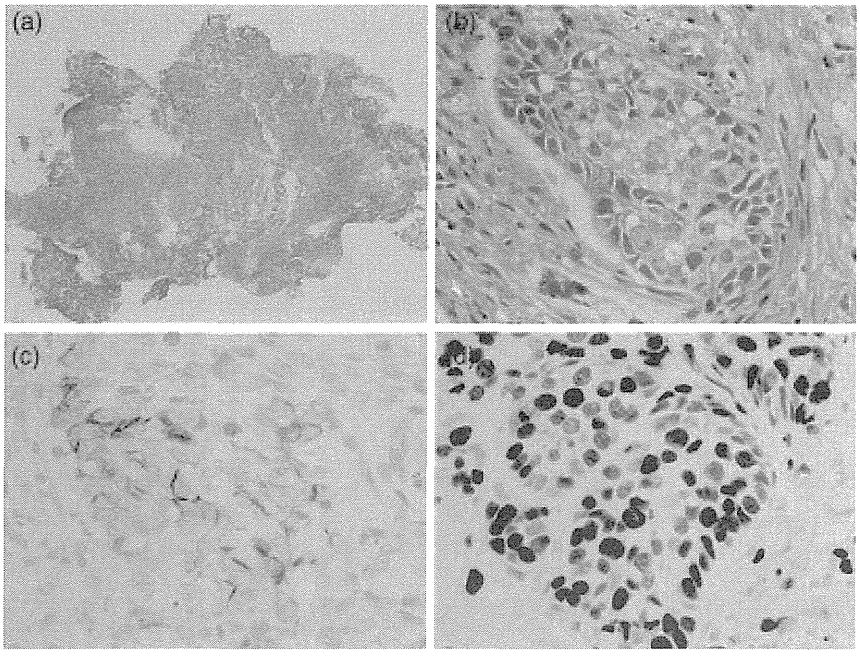

Figure 2. Morphological and immunohistochemical features of a biopsy specimen (Case 1). The low-powered view shows an irregular organoid nesting structure (a, H&E, ×4). In the high-powered view, the tumor consists of two types of cells, cells with nuclei with a fine chromatin pattern and larger cells with prominent nucleoli, and tumor cells show incomplete rosette and intercellular cleft formation (b, H&E, ×40). Immunohistochemically, NCAM (c, ×40) is positive and the Ki-67 labeling index is high (d, ×40).

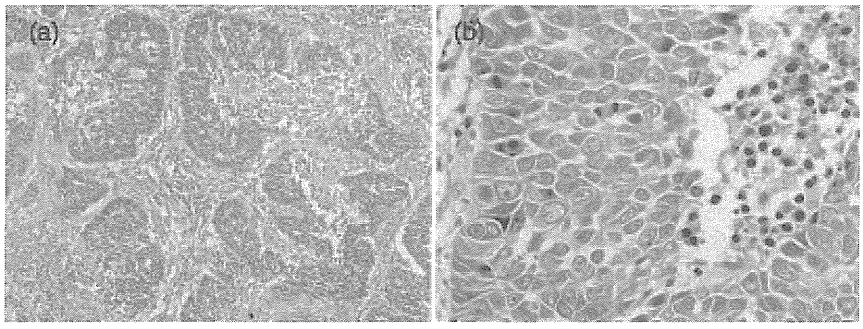

Figure 3. The morphology of a surgically resected tumor of Case 1. Tumor cells form an organoid nesting structure with necrosis (a, H&E, ×4) and show the features of non-small cell lung cancer with large nuclei with prominent nucleoli (b, H&E, ×40).

HNSCNECs. Among them, a fine nuclear chromatin pattern was the most frequent finding. These six biopsy specimens had a Ki-67/MIB1 labeling index from 56.6 to 98.7% (median: 70.6%).

The comparisons between histological findings of the biopsy specimen and the resected one in each case are shown in Fig. 4 and Table 5. Fine chromatin in biopsy specimens generally has changed to be rather course chromatin in resected specimens, and peripheral palisading, rosette formation, trabecular arrangement and organoid pattern have become clearly visible in four cases out of six.

DISCUSSION

Using our HNSCNEC criteria (9), we selected 38 cases from all lung biopsy specimens obtained in our hospital from 2002 to 2009. As shown in Table 2, various clinical characteristics of the 38 cases corresponded well with those of LCNEC cases, which have been already reported by many previous papers using surgically resected LCNEC (1,11–18). For example, CT findings have already revealed that LCNEC is peripherally located and has a solid mass with a lobulated margin (19). In this study, the serum pro-GRP level was elevated in 13 (35.1%) of 37 cases, but no report about the frequency of elevated serum pro-GRP in LCNEC has appeared in the literature. Previous investigations reported that the frequency of the elevated serum pro-GRP level is 68% in SCLC and 4.2% in NSCLC (20). The frequency of the elevated serum pro-GRP level might be low compared with that of SCLC.

The majority of the 38 cases had pathologically Stage IV disease and many patients died within 1 year after the lung

Table 5. Comparison of histological findings between biopsy specimen and resected ones (biopsy/resection) in Cases 1–6 in Table 2

Finding	Cytoplasm[a]	Nucleus[a]		Structure[a]					
	Wide	Fine chromatin	Nucleolar prominence	Peripheral palisading	Rosettes	Trabecular	Organoid	Discohesive	Necrosis
Case 1*	2/1	2/0[b]	2/2	2/1	2/2	0/0	3/3	1/3	0/2
Case 2	1/1	2/1	1/2	1/3	1/1	0/3	1/3	1/1	0/0
Case 3**	1/2	2/0	2/2	0/0	0/3	0/1	0/1	0/1	0/1
Case 4	2/2	2/1	1/2	0/0	1/2	0/0	1/3	1/0	2/1
Case 5**	2/3	3/1	0/2	0/2	0/1	0/1	0/2	3/1	2/2
Case 6	3/3	3/1	0/1	0/0	0/0	0/0	1/2	1/0	0/1

[a]Each numeral indicates: [b]0, not observed; 1, partially or focally observed; 2, easily and/or widely observed; 3, remarkably observed. Underline indicates that a difference of 2 or 3 degrees exists between evaluation numerals of biopsy specimen and resected one.
*Findings shown in Figs 2 and 3.
**Findings shown in Fig. 4.

biopsy in this study. LCNEC is a highly malignant neoplasm and previous comparative studies using surgically resected high-grade neuroendocrine carcinoma cases revealed that the prognosis of LCNEC is similar to that of SCLC (16,18,21). Of the 38 cases in this study, 6 were clinical TNM Stage I, II or IIIA, which then underwent surgery after biopsy and the diagnosis was pathologically confirmed as LCNEC using surgically resected tumor specimens. These findings suggest that our HNSCNEC criteria are applicable in practical use for the diagnosis of LCNEC using biopsy specimens.

Among high-grade neuroendocrine carcinoma of the lung, the overall clinicopathological features of LCNEC, including unresectable cases, is still uncertain compared with those of SCLC (22). As it is extremely difficult to make a final diagnosis of LCNEC using only biopsy and/or cytology specimens, the true frequency of LCNEC is undetermined. As the results of a study using surgically resected LCNEC cases, the frequency has been reported to be 1.6–3.1% (7,11,13). If all 38 cases in our study were compatible with LCNEC, the true frequency of LCNEC would be 3.7% (38 out of 1040 biopsies). In the Japanese lung cancer registry study using a large number of surgical and non-surgical cases in 2002, the proportions of adenocarcinoma, squamous cell carcinoma and small cell carcinoma were 56.7, 25.7 and 9.2%, respectively (23). In our biopsy series, these proportions were 49.8, 30.6 and 11.6%, which showed a similar tendency to those of the Japanese lung cancer registry study, although the number is small; therefore, the real frequency of LCNEC might be over 3.7%.

In 1991, Travis et al. (1) proposed a category of LCNEC that was adopted in the WHO classification of lung cancer in 1999 and 2004 (2,3); however, these criteria are applicable only to surgically resected specimens, not to biopsy specimens. Biopsy specimens are too small to have enough morphological information and to count the number of mitoses; therefore, we modified the WHO criteria of LCNEC and proposed new criteria for diagnosing LCNEC using biopsy specimens (9). In our criteria, the immunohistochemical Ki-67/MIB1 labeling index was used instead of the mitotic count. Our study clearly showed that it was very difficult or impossible to count mitoses in small biopsy specimens, but immunohistochemical Ki-67/MIB1 labeling indices could be useful for evaluating the proliferation activity. Fortunately, for 7 of 38 biopsy specimens the number of mitoses could be counted and these specimens were elucidated to have more proliferative activity than biopsy specimens with an impossible mitosis count. In Stage I NSCLCs, the mean Ki-67/MIB1 labeling index is 19.3% (24), and the prognosis of NSCLC patients is reported to differ between the '20% or higher' group and the 'lower than 20%' group of the Ki67/MIB1 labeling index (25). When compared with the positivity of the Ki-67/MIB1 labeling index of NSCLC, the labeling index of our biopsy specimens in this study was quite high, which suggests that high proliferative activity is one of the characteristic features of LCNEC (26).

In the WHO criteria, large cell morphology is one of the most important criteria for LCNEC, such as large tumor cell size with abundant cytoplasm as well as vesicular nuclei and prominent nucleoli (2,3). Our criteria of HNSCNEC (9) also followed the WHO criteria; however, the recognition of this large cell morphology is problematic among pathologists. In previously published papers discussing the interobserver variability of SCLC and LCNEC, the cytologic features of a nuclear/cytoplasmic ratio may be recognized arbitrarily or not quantitatively among diagnostic pathologists (4,5,17,27). It is also reported that there are borderline cases between LCNEC and SCLC in high-grade neuroendocrine carcinoma (17). In this study, about one-third of cases were diagnosed as high-grade neuroendocrine carcinoma, but there was a hesitation to diagnose them as LCNEC, because of the cytological similarity to SCLC. Previous studies using surgically resected tumors revealed that LCNEC and SCLC have distinct characteristic morphological features; namely, a large tumor cell size, peripheral palisading and prominent nucleoli

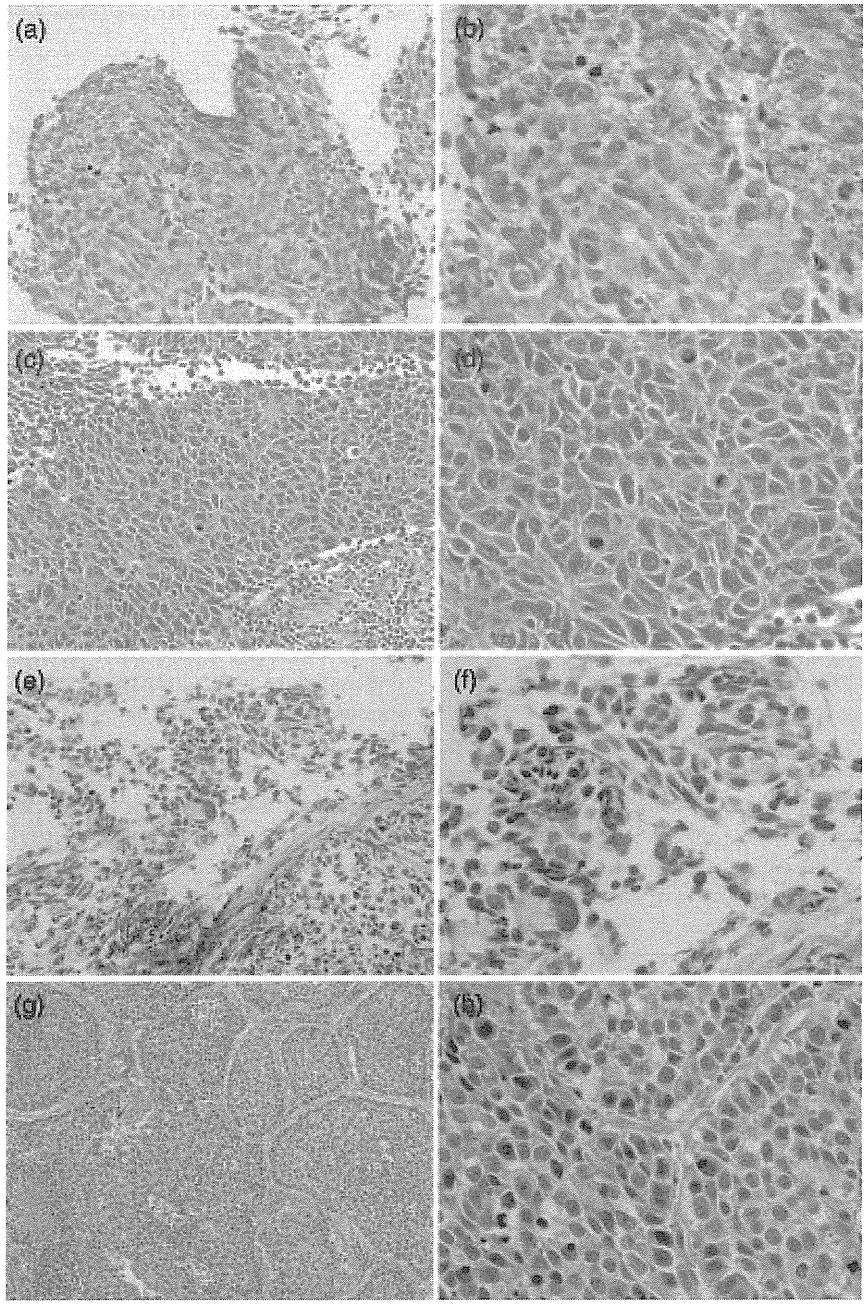

Figure 4. Histological comparison between biopsy and resected specimens in two of the resected six cases. In Case 3 (a–d), the biopsy specimen shows fine chromatin architecture with visible nucleoli and an obscure rosette pattern (a, H&E, ×20; b, H&E, ×40). However, in the resected specimen, a large number of rosettes are observed (c, H&E, ×20; d, H&E, ×40). The chromatin is coarse and the cytoplasm is abundant. In Case 5 (e–h), the biopsy specimen is crushed and neither the neuroendocrine structure nor nucleoli can be recognized (e, H&E, ×20; f, H&E, ×40). However, in the resected specimen, peripheral palisading, organoid nesting and nucleoli are clearly observed (g, H&E, ×10; h, H&E, ×40).

are characteristic of LCNEC, and a small cell size, high nuclear/cytoplasmic ratio and fine chromatin pattern are characteristic findings of SCLC (1–3,17,18); however, these morphological features are known to overlap among LCNEC and SCLC (1,17,18). As shown in Table 3, morphological analysis of 38 biopsy specimens revealed that the incidence of the specimen with a fine chromatin pattern was the highest, and those with intracellular cleft and prominent nucleoli followed in a decreasing order. Therefore, the morphological fine chromatin pattern is not only seen in surgically resected SCLC, but is also a characteristic finding of LCNEC in biopsy specimens as shown in Figs 2 and 4, Tables 3 and 5. Even in surgically resected LCNEC, the appearance of tumor cells with fine nuclear chromatin has been

Table 6. Comparison among diagnostic criteria of WHO (3), our former proposal (9) and the present study for LCNEC

Findings	WHO 2004 (3)	Igawa et al. (9)	Present study
Applied specimen	Resected	Biopsy	Biopsy
Differentiation			
Histological differentiation without SCLC morphology, glandular and squamous differentiation	EA, Ess	EA, Ess	EA, Ess
Cell size			
Large cell size and low nuclear/cytoplasmic ratio	EA, Ess	EA, Ess	EA, Ess
Neuroendocrine differentiation			
Immunostain positive for at least one of neuroendocrine markers	EA, Ess	EA, Ess	EA, Ess
Proliferating activity			
Mitosis (11 or greater/mm^2)	EA, Ess	DA	DA, nd
Ki67/MiB-1 labeling index (>40%)	nd	EA, Ess	EA, Ess
Nuclear findings			
Nucleoli (frequent and prominent)	EA, LS	EA, LS	EA, LS
Chromatin pattern (vesicular, coarse or fine)[a]	EA, Ess (vesicular or coarse chromatin pattern is common)	EA, Ess	EA (fine chromatin pattern is common)
Neuroendocrine morphology			
Organoid nesting, palisading and rosettes, trabecular	EA, Ess	CA, SF	CA, SF
Others			
Necrosis	EA, common	CA, SF	CA, SF
Cellular atypia	nd	Moderate to severe, Ess	nd
Intercellular space (cleft)	nd	EA, SF	EA, SF

LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; EA, easily assessed; Ess, essential; DA, difficult to be assessed; nd, not determined, LS, lacking sometimes; CA, can be assessed sometimes; SF, supportive findings.
[a]Ref. (2) and Table 1.08 in ref. (3).

noted (1,18). As our resected cases were found to show a chromatin pattern changed from the fine pattern in the biopsy specimen to a coarse or vesicular pattern (Fig. 4 and Table 5), it is supposed that the different fixative conditions might have some effects on the chromatin morphology of tumor cells. The greatest difference might be due to a rapid start and full immersion in formalin soon after taking a biopsy specimen, in contrast to a delayed start and immersion in a relatively small ratio of fixative amount/specimen size for a resected specimen, resulting in fine chromatin morphology and a coarse or vesicular one, respectively. Furthermore, as another histological finding, intercellular cleft might reflect poor intercellular connection and might be one of the characteristics of LCNEC.

In LCNEC, the appearance of neuroendocrine morphology such as organoid nesting, palisading, rosette and trabeculae has been frequently observed and their frequencies are reported to be 90.9, 59.1, 72.7 and 31.8%, respectively (18). In contrast, Figs 2 and 4 revealed that the neuroendocrine morphology was relatively difficult to recognize in biopsy specimens. This may be attributed to the small size of specimens. Therefore, immunohistochemical detection of a neuroendocrine marker is a more reliable method to identify the neuroendocrine features of biopsy specimens. In a previous report, synaptophysin was frequently positive at the rate of 77% in LCNEC, and chromogranin A and CD56 followed (17). In our study, NCAM was the most frequently immunopositive neuroendocrine marker and chromogranin A followed after synaptophysin. This may be explained by the difference of the pattern of immunostaining because NCAM tends to stain tumor cells diffusely compared with chromogranin A or synaptophysin. For immunohistochemical evaluation of neuroendocrine features in both surgically resected or biopsy samples, immunohistochemistry using three neuroendocrine markers is necessary.

Although the cases in the early stage can be resected and histologically confirmed, the cases in an advanced stage should receive chemotherapy without histologically examining resected specimens. Then the criteria of HNSCNEC may give the chance for lesions that have the possibility of being LCNEC to be treated as inoperable advanced probable LCNEC cases. For this reason some papers have reported that the clinical efficacy of chemotherapy for LCNEC is comparable with that for SCLC (7–10); the diagnosis of

Table 7. Proposed diagnostic criteria for possible LCNEC using biopsy specimens

Major points
1. Poorly differentiated NSCLC with neither acinar nor squamous differentiation
(a) Tumor cell contains a nucleus larger than the size of three small resting lymphocytes, and low nuclear/cytoplasmic ratio or abundant cytoplasm
(b) Tumor nucleus with a fine chromatin pattern and/or prominent nucleoli
2. Ki-67/MIB1 labeling index >40%
3. Positive immunostaining for one or more neuroendocrine markers (NCAM, chromogranin A and synaptophysin)
Minor points
1. Neuroendocrine morphology (organoid nesting, peripheral palisading[a], rosettes, trabecular architecture)
2. Frequent massive necrosis
3. Intercellular space (cleft) or discohesiveness

[a]Peripheral palisading is mentioned as 'basal palisading' in Table 1; both stand for a similar finding.

HNSCNEC as a surrogate of LCNEC or as a probable LCNEC would help these lesions to receive more appropriate therapy. When the criteria of HNSCNEC work, the number of patients who receive chemotherapy for LCNEC, comparable to that for SCLC, might increase by 0.6–2.1% of the number of all lung carcinomas. This estimation is according to the comparison between the reported frequency (1.6–3.1%) of LCNEC (7,11,13) and the incidence (3.7%, 38 out of 1040 lung biopsies positive for cancer) of HNSCNEC in our hospital, which has already been discussed.

WHO criteria (3), our previous criteria (9) and our new criteria have three common essential points as indicated in the upper one-third of Table 6; and there is one more essential point on proliferating activity. In our new criteria, the Ki67/MIB1 labeling index is adopted instead of mitotic counting. Then our new criteria can be shown as in Table 7. Summarizing the data obtained in this study using biopsy specimens, we simplified our previous criteria for HNSCNEC and proposed new diagnostic criteria suitable for possible LCNEC (28). In the new criteria (Table 7), the major points are essential for diagnosing LCNEC using biopsy specimens. In addition to the major points, the findings due to additional minor points would increase the possibility of an LCNEC diagnosis. We expect these new criteria to be used in routine surgical pathology and to facilitate the clinicopathological study of high-grade neuroendocrine carcinoma, especially LCNEC.

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Conflict of interest statement

None declared.

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Combined Treatment with Erlotinib and a Transforming Growth Factor-β Type I Receptor Inhibitor Effectively Suppresses the Enhanced Motility of Erlotinib-Resistant Non–Small-Cell Lung Cancer Cells

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Introduction: Despite an initial dramatic response to the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib, the majority of non–small cell lung cancer (NSCLC) patients with EGFR-activating mutations develop acquired resistance. Therefore, there is an urgent need to elucidate the unknown mechanisms and biological behaviors of EGFR TKI–resistant lung tumors. We investigated the motility of EGFR TKI–resistant cells, as these characteristics are relevant to cancer metastasis.

Methods: Erlotinib-resistant PC-9ER cells were generated from PC-9 NSCLC cells, which harbor an EGFR-activating mutation, and used in this study. We investigated the involvement of the transforming growth factor beta (TGF-β) pathway in cell motility, and tested the effects of erlotinib and TGF-β type I receptor (RI) inhibition on cell motility.

Results: PC-9ER cells displayed enhanced motility resulting from autocrine activation of the TGF-β pathway. Increased TGF-β2 secretion resulting from TGF-β2 up-regulation at the transcriptional level was suggested to be responsible for the phosphorylation of Smad2 and the subsequently elevated transcriptional regulatory activity in PC-9ER cells. The motility of PC-9ER cells was suppressed by treatment with either the TGF-βRI inhibitor LY364947 or erlotinib, and greater suppression was observed when used in combination. LY364947 or erlotinib exerted no growth-inhibitory effects, suggesting that motility and growth are driven by different signaling pathways in PC-9ER cells.

Conclusions: Our results imply that blockade of the TGF-β signaling pathway combined with continuous EGFR TKI treatment will be beneficial in preventing metastasis in patients with EGFR TKI–resistant NSCLC without the EGFR T790M resistance mutation.

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The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib have been demonstrated to be effective in non–small-cell lung cancer (NSCLC) patients with EGFR-activating mutations.[1–6] However, the majority of responders eventually develop acquired resistance to EGFR TKIs.[3] Although studies have investigated the cause of acquired resistance to EGFR TKIs,[7–14] the characteristics of EGFR TKI–resistant tumors such as cell motility that play a pivotal role in the process of metastasis have been poorly investigated.

Recent studies suggested that both genetic alterations within cancer cells[7–9,12,14] and the activation of the tumor microenvironment such as the induction of the epithelial-mesenchymal transition (EMT) could confer resistance to EGFR TKIs.[15,16] EMT is known to be induced by activation of the transforming growth factor beta (TGF-β) pathway, which plays an important role in tumor invasiveness and metastasis by directly promoting the motility and/or migratory ability of various types of cancers including lung cancer.[17] TGF-β ligands bind and activate the TGF-β receptor complex composed of the type II (TGF-βRII) and type I subunits (TGF-βRI), which phosphorylate Smad2 and Smad3. Activated Smad2/3 forms transcriptional complexes with Smad4 and other transcriptional factors and regulates the transcription of genes associated with tumor-promoting effects.[17] It has been reported that the high expression level of TGF-β in several tumors correlates with tumor progression and metastasis.[18] TGF-β1 expression was reported to be significantly higher in NSCLC patients with lymph node metastasis than in those without lymph node metastasis.[19] It has also been reported that TGF-β1 expression levels in patients with stage III NSCLC are significantly higher than those in patients with stage I and II NSCLC, and the overall survival of patients with high TGF-β1 levels was significantly shorter than that of patients with low TGF-β1 levels.[19] Blockade of TGF-β signaling by

TGF-βRI inhibitors or soluble TGF-β receptor antagonists decreased tumor invasion and migration in preclinical models,[20,21] providing a rationale for a therapeutic approach to prevent metastasis based on the inhibition of TGF-β signaling. EGFR TKI treatment has also been suggested to inhibit TGF-β–induced cell motility,[22] which leads to the hypothesis that the combination of EGFR TKIs and TGF-β inhibitors may be more beneficial for preventing metastasis. In this study, we investigated the involvement of the TGF-β pathway in the motility of erlotinib-resistant NSCLC cells and the effects of erlotinib and TGF-βRI inhibition on erlotinib-resistant NSCLC cells.

MATERIALS AND METHODS

Cell Culture and Reagents

The human NSCLC (adenocarcinoma) cell line PC-9, which harbors an EGFR-activating mutation (exon 19 deletion, E746-A750del), was provided by Dr. Koizumi (National Cancer Center Hospital, Tokyo, Japan). The human NSCLC (squamous cell carcinoma) cell line EBC1, which is characterized by *MET* proto-oncogene amplification, was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). All cell lines used in this study were maintained in RPMI1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) in humidified air containing 5% carbon dioxide at 37°C. Erlotinib was supplied by F. Hoffmann-La Roche (Basel, Switzerland). Gefitinib (Tocris Bioscience, Ellisville, MO), LY364947 (Sigma, St. Louis, MO), and PHA-665752 (Tocris Bioscience) were purchased from the indicated companies. TGF-β2 was purchased from R & D Systems (Minneapolis, MN).

Establishment of the Erlotinib-Resistant Cell Lines

PC-9 cells were exposed to increasing concentrations of erlotinib (0.1–10 μmol/l) for 15 months. Several clones were subcloned by limiting dilution, and we selected two clones (PC-9ER1 and PC-9ER4) for this study after we confirmed that they acquired resistance to erlotinib.

In Vitro Growth Inhibition Assay

The growth-inhibitory effects of erlotinib, gefitinib, LY364947, and PHA-665752 were examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described previously.[23] In brief, a 180-μl volume of an exponentially growing cell suspension was seeded into each well of a 96-well plate (Corning Coaster, Cambridge, MA) containing 10% FBS medium and incubated for 24 hours. The cells were exposed to 20 μl of each inhibitor at various concentrations and further cultured at 37°C in a humidified atmosphere for 72 hours. After the culture period, 20 μl of MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well, and the plates were incubated for 4 hours at 37°C. After centrifuging the plates, the medium was aspirated from each well, and 200 μl of DMSO was added to each well to dissolve the formazan. The growth-inhibitory effect of each inhibitor was assessed spectrophotometrically (Model 680 microplate plate reader; Bio-Rad Laboratories, Hercules, CA).

Immunoblot Analysis

The cultured cells were washed twice with ice-cold phosphate-buffered saline and lysed in M-PER mammalian protein extraction reagent with an EDTA-free Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Pierce Chemical Co., Rockford, IL). Equal amounts of protein (20 μg) in whole-cell lysate (as measured by using the BCA protein assay reagent [Pierce Chemical Co.]) were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% skim milk or 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) for 1 hour at room temperature and then incubated overnight with primary antibodies in TBST containing 5% skim milk or 5% BSA at 4°C. After the membranes were washed thrice with TBST, they were incubated with secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature and then subjected to three washes with TBST. Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.), and the images were captured using the ImageQuant LAS4000 system (GE Healthcare, Piscataway, NJ). Densitometric analysis was performed using ImageQuant TL software (GE Healthcare). The antibodies used for this analysis are listed in Supplementary Table S1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A361).

Wound-Closure Assay

Cells (3×10^5 cells/well) were seeded into 6-well plates (Corning Coaster). Cell motility was determined by measuring the movement of cells to close an artificial wound created with a 200-μL pipette tip (time point 0 hour) when the cells reached confluency. Afterward, the cells were washed with FBS-free medium and then incubated in fresh 1% FBS medium with or without inhibitors. Wound closure was monitored by microscopy at the indicated time points.

Transwell Assay

The transwell assay was performed according to the method of Bakin et al.[24] Cells (1.5×10^5 cells/well) were seeded in 0.1% FBS medium in the upper chamber of a 5-μm pore filter unit (Corning Coaster) and incubated in 1% FBS medium with or without the inhibitors. Twenty-four hours later, cells that had migrated through pores and reattached to the bottom of lower chamber were trypsinized, and the cell number was counted using a Coulter Z1 particle counter (Beckman Coulter, Brea, CA).

Luciferase Assay

Cells (5×10^4 cells/well) were seeded in 24-well plates (Corning Coaster). After incubation for 48 hours, cells were transfected with 1 μg/well of either $p(CAGA)_{12}$-Lux or p3TP-Lux Smad-dependent reporter plasmid containing Firefly luciferase using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was normalized by cotransfecting 16 ng/well of pRL-CMV (Promega, Madison, WI), which contains the cytomegalovirus enhancer, to provide stably high expression of *Renilla*