Which is the Better Prognostic Factor for Resected Non-small Cell Lung Cancer

The Number of Metastatic Lymph Nodes or the Currently Used Nodal Stage Classification?

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Introduction: This retrospective study was conducted to evaluate the prognostic significance of the number of metastatic lymph nodes (nN) in resected non-small cell lung cancer (NSCLC) in comparison with the currently used pathologic nodal (pN) category in the staging system.

Methods: A total of 1659 patients who underwent potentially curative resection for NSCLC from 2000 to 2006 were included in this study. The association between the nN and survival was explored, and the results were compared with those using the location-based pN stage classification.

Results: The patients were divided into four categories according to the number of metastatic nodes: nN0, absence of metastatic nodes; nN1, metastasis in one to two nodes; nN2, metastasis in three to six nodes; and nN3, metastasis in seven or more nodes. The 5-year overall survival for nN0, nN1, nN2, and nN3 was 89.2%, 65.1%, 42.1%, and 22.4%, respectively ($p < 0.001$). The nN category could be used to subdivide pN1 and pN2 patients into two (nN1 and nN2) and three (nN1, nN2, and nN3) prognostically distinct subgroups, respectively. Multivariate analysis showed the nN category was an independent prognostic factor for resected NSCLC. The difference in overall survival between pN1 and pN2 was not significant (55.4% versus 47.8%, $p = 0.245$). Patients in each nN category could not be subdivided into different prognostic subgroups according to the pN classification.

Conclusions: The nN category in this study was shown to be a better prognostic determinant than the location-based pN stage classification.

Key Words: Non-small cell lung cancer, Lymph node metastasis, Prognosis.

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The accurate assessment of lymph node involvement is crucial for the diagnosis and treatment of non-small cell lung cancer (NSCLC). Recently, the seventh edition of the tumor-node-metastasis (TNM) classification for NSCLC has been accepted with some modifications in comparison with the sixth edition.[1] However, the node (N) descriptor in the new classification remains the same as in the previous edition and depends solely on the anatomic extent of lymph node involvement despite the change in the nodal map.

Patients suffering from pathologic N1 or N2 disease in NSCLC have long been known to exhibit prognostic heterogeneity.[2–13] This has indicated that it is necessary to refine the currently used pathologic N (pN) stage classification and has justified attempts to identify alternative nodal classification methods. In some other solid tumors, such as breast, gastric, and colorectal cancer, the number of metastatic lymph nodes has been considered in the TNM staging system.[14] Recently, the number of metastatic lymph nodes (nN), when classified into several categories, has been shown to be a prognostic factor for resected NSCLC.[9,15] However, to date, it is still unknown whether the nN category or the pN stage classification is the better prognostic factor. In this study, we retrospectively evaluated the association between the nN category and the prognosis of resected NSCLC and compared the results with the classic pN stage classification.

PATIENTS AND METHODS

Patient Selection

A total of 2333 consecutive patients with NSCLC who underwent surgery at the National Cancer Center Hospital, Tokyo, from January 2000 to December 2006 were examined retrospectively. The Institutional Review Board approved this retrospective study, and informed consent from patients was waived.

All the patients received a thorough work-up preoperatively, including computed tomography (CT) scan, chest radiograph, blood test, and positron emission tomography (PET), if necessary, to evaluate their eligibility for surgery. Patients who were identified to have distant metastasis or clinical N2 (cN2) diseases preoperatively were excluded from

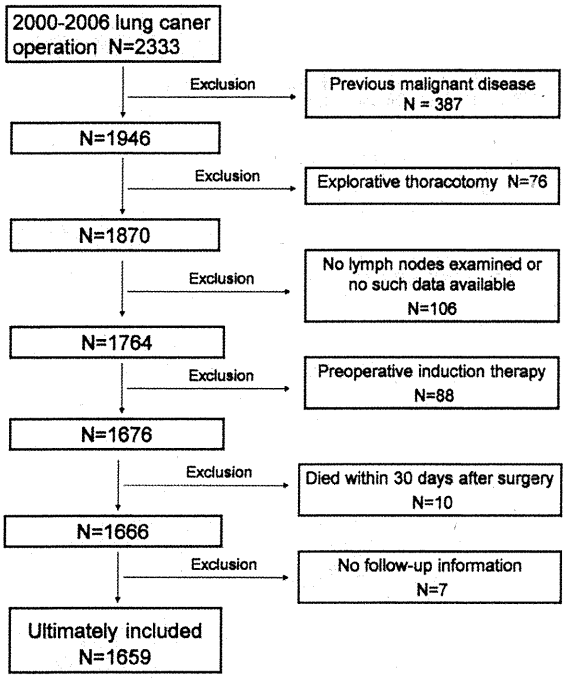

FIGURE 1. Patients included in this study.

surgery. Clinical N2 disease was suspected when mediastinal lymph node enlargement was present with a minimal diameter of 1.0 cm or more on the CT scan. If the following PET scan or mediastinoscopy was positive, we considered it as cN2 disease and excluded from surgery. PET scan and mediastinoscopy were used only for suspected cN2 disease rather than for all patients.

Patients with a prior history of malignant disease or induction therapy before surgery and those who underwent explorative thoracotomy were not included in this study. In addition, those who died within 30 days after surgery, those for whom no lymph node was retrieved or no such data were available, and those for whom follow-up information was unavailable were also excluded. Ultimately, a total of 1659 patients were included in this study (Figure 1).

Procedure Performed

Procedures performed for the affected pulmonary consisted of wedge resection/segmentectomy, lobectomy, bilobectomy, and pneumonectomy. Wedge resection or segmentectomy was performed only for the high-risk patients, combined with minimal lymph node dissection, sometimes maybe around the hilum only. For all the other patients, we performed lymph node dissection based on the lobe-specific patterns of nodal metastases.[16,17] When the cancer located in right upper lobe or left upper division, the hilar lymph nodes and upper mediastinal lymph nodes were dissected. The upper mediastinal lymph nodes indicate highest mediastinal nodes, paratracheal nodes, pretracheal nodes, and tracheobronchial nodes in right side and aortopulmonary window nodes, paraaortic nodes, and tracheobronchial nodes in left side. If intraoperative frozen section of the lymph node in either hilum or upper mediastinum was positive, we performed subcarinal dissection, which was omitted otherwise. For the tumor located in right middle lobe or left lingular segment, the lymph nodes in both upper mediastinum and subcarina were routinely dissected in addition to the hilum. When the tumor located in lower lobe, the hilar and lower mediastinal lymph nodes (including stations 7, 8, and 9) were dissected. If intraoperative pathologic examination of either the hilar or the lower mediastinal lymph node was positive, we performed additionally upper mediastinum dissection, which was omitted otherwise.

Data Collected and Statistical Analyses

The data collected included the patient demographics, surgical procedures, and pathologic reports including the histologic type, T stage, and the number and location of all the malignant and benign lymph nodes. The TNM classification was based on the sixth edition of the TNM staging system.[18]

We choose overall survival (OS) and disease-free survival (DFS) as the end points. OS was the time between surgery and death from any cause. DFS was the time from surgery to locoregional or distant relapse of lung cancer, and if without relapse, any deaths due to causes other than lung cancer would be censored. Continuous variables are expressed as the mean ± SD. The associations between variables were analyzed by either χ^2 test or Mann-Whitney and Wilcoxon tests. Survival curves were generated by the Kaplan-Meier method, and differences in survival among subgroups were examined by the log-rank test. A multivariate analysis was performed using Cox proportional hazards models to examine the association between survival and potential prognostic factors. A probability value of less than 0.05 was considered to be significant. All statistical calculations were performed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL).

RESULTS

Patient Characteristics

The 1659 patients in this study consisted of 962 men and 697 women with a mean age of 63.8 ± 10.0 years (range, 26–89 years). The characteristics of the patients are shown in Table 1. The most frequent procedure performed was lobectomy (86.4%, n = 1434), followed by wedge resection/segmentectomy (5.4%, n = 90). Adenocarcinoma was found in 78.3% (n = 1299) of the patients, and the second most common histologic type was squamous cell carcinoma (17.4%, n = 288). A positive surgical margin was confirmed in 95 (5.7%) patients by pathologic examination. A total of 102 (6.1%) patients were classified as stage IIIb due to pleural dissemination or separate tumor nodule(s) in the same lobe identified during the operation, rather than pN3 disease. Stage IV disease (0.4%, n = 7) consisted of separate tumor nodule(s) in a different ipsilateral lobe identified during surgery.

TABLE 1. Patient Characteristics

Patient Characteristics	*N* (%)
Age (range)	63.8 ± 10.0 (26–89)
Gender	
Male	962 (58%)
Female	697 (42%)
Surgical procedure	
Wedge resection/segmentectomy	90 (5.4%)
Lobectomy	1434 (86.4%)
Bilobectomy	60 (3.6%)
Pneumonectomy	75 (4.5%)
Surgical margin	
Positive	95 (5.7%)
Negative	1564 (94.3%)
Histological type	
Adenocarcinoma	1299 (78.3%)
Squamous cell carcinoma	288 (17.4%)
Large cell carcinoma	53 (3.2%)
Adenosquamous carcinoma	19 (1.1%)
pT stage	
pT1	953 (57.4%)
pT2	500 (30.1%)
pT3	109 (6.6%)
pT4	97 (5.8%)
pN stage	
pN0	1203 (72.5%)
pN1	213 (12.8%)
pN2	243 (14.6%)
UICC stage (sixth edition)	
Ia	813 (45.0%)
Ib	302 (18.2%)
IIa	65 (3.9%)
IIb	156 (9.4%)
IIIa	214 (12.9%)
IIIb	102 (6.1%)
IV	7 (0.4%)

UICC, International Union Against Cancer.

Lymph Node Metastasis and Definition of nN Category

The mean number of lymph nodes retrieved from each patient was 15.9 ± 9.5 (range, 1–79). Lymph node metastasis was seen in 456 patients. The mean number of metastatic lymph nodes was 4.11 ± 4.43 (range, 1–29). Among the patients with nodal metastasis, 213 were identified as pN1 and 243 were pN2. The distribution of the number of metastatic lymph nodes in pN1 and pN2 is shown in Figure 2. The pN2 stage had more lymph node metastasis than pN1 (5.74 ± 5.35 versus 2.26 ± 1.71, $p < 0.001$) (Table 2).

The last follow-up visit was in August 2007, and the median follow-up duration was 30.0 months (range, 1–88 months). During the follow-up period, 386 cases suffered from locoregional or distant recurrence of lung cancer, and 214 cases died due to any cause. The 5-year OS and DFS rates for the overall population were 78.9% and 68.4%, respectively.

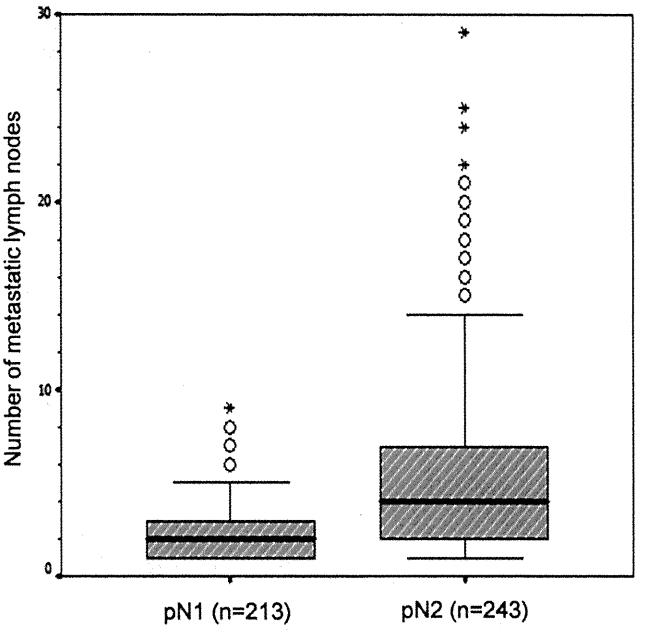

FIGURE 2. Distribution of the metastatic lymph nodes in pN1 and pN2 stage.

TABLE 2. Comparison of Number of Metastatic Lymph Nodes between pN1 and pN2 Stage

pN Stage	Number of Metastatic Lymph Nodes (Mean ± SD)	*p*
pN1	2.26 ± 1.71	<0.001
pN2	5.74 ± 5.35	

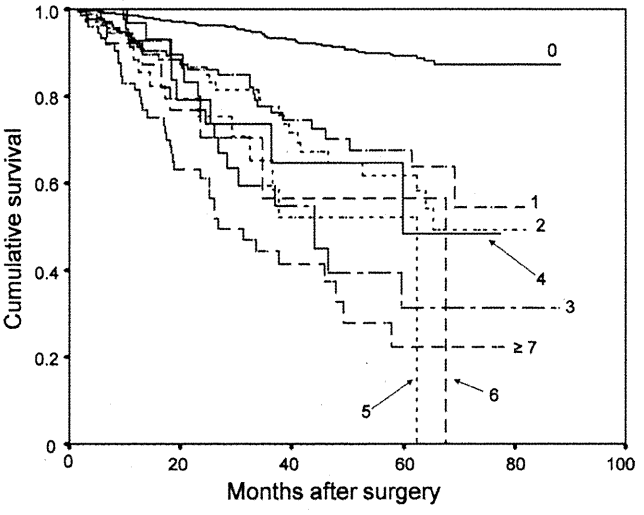

FIGURE 3. The survival curves according to different number of metastatic lymph nodes.

OS deteriorated with an increase in the number of metastatic lymph nodes (Figure 3). We classified the patients into four nN categories, and the survival curves that were

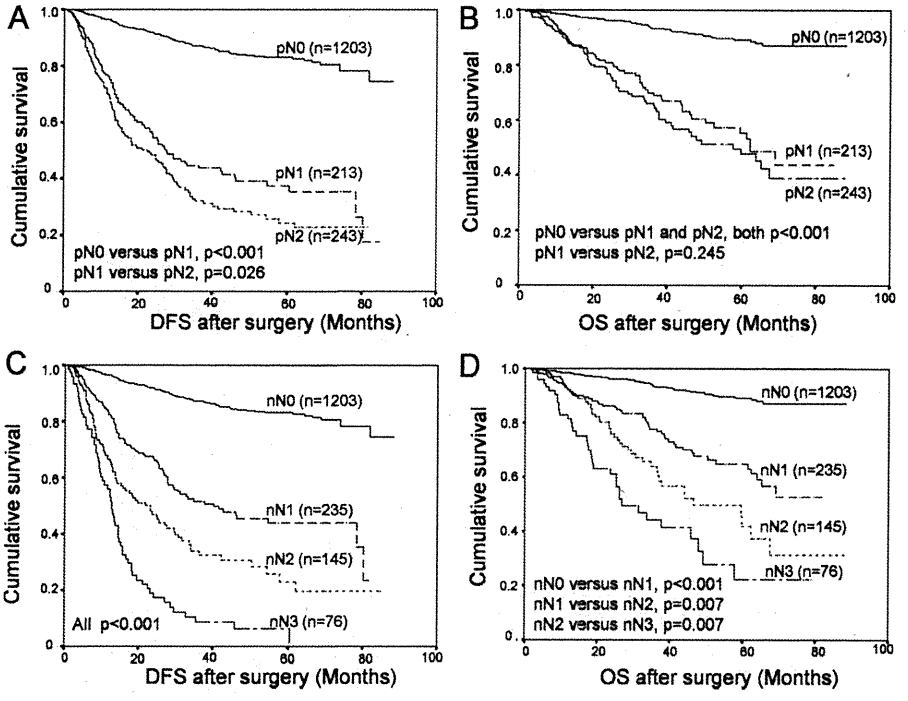

FIGURE 4. Disease-free survival (DFS) and overall survival (OS) according to pN stage classification and nN category. *A*, DFS curves according to pN stage classification. Five-year DFS rate for pN0, pN1, and pN2 was 83.2%, 37.3%, and 24.5%, respectively. *B*, OS curves according to pN stage classification. Five-year OS rate for pN0, pN1, and pN2 was 89.2%, 55.4%, and 47.8%, respectively. *C*, DFS curves according to nN category. Five-year DFS rate for nN0, nN1, nN2, and nN3 was 83.2%, 44.1%, 23.0%, and 6.5%, respectively. *D*, OS curves according to nN category. Five-year OS rate for nN0, nN1, nN2, and nN3 was 89.2%, 65.1%, 42.1% and 22.4%, respectively.

close to each other were grouped into a single category. Finally, the four nN categories were defined as follows: nN0, no lymph node metastasis; nN1, metastasis in one to two nodes; nN2, metastasis in three to six nodes; and nN3, metastasis in seven or more lymph nodes.

Prognostic Significance of nN Category and Comparison with pN Stage Classification

The OS and DFS in each pN stage classification and nN category were explored. Patients without lymph node metastasis (pN0 and nN0) had the most favorable prognosis, with 5-year OS and DFS rates of 89.2% and 83.2%, respectively. There was a significant difference between pN1 and pN2 disease with regard to DFS (5-year DFS rate: 37.3% versus 24.5%, $p = 0.026$), but the difference in OS was not significant (5-year OS rate: 55.4% versus 47.8%, $p = 0.245$) (Figures 4*A*, *B*). DFS and OS according to the nN category are shown in Figures 4*C*, *D*. The survival curves showed clear differences in OS and DFS for each of the nN categories (5-year OS rate for nN0, nN1, nN2, and nN3 was 89.2%, 65.1%, 42.1%, and 22.4%, respectively, $p < 0.001$; 5-year DFS rate was 83.2%, 44.1%, 23.0%, and 6.5%, respectively, $p < 0.001$).

A validation of the results of the nN category in terms of OS across each pT stage was performed. The results were showed in Figure 5. Although the differences between each pair of nN categories were not always significant, there was a clear tendency of deterioration of the OS from nN0 to nN3 subgroup, and the curves were apart from each other in pT1 and pT2 patients. There were similar results in terms of DFS (data not shown).

When pN1 patients were subdivided into nN1, nN2, and nN3 subgroups, there was a significant difference between the nN1 and nN2 subgroups with regard to both OS (5-year OS rate: 61.5% versus 35.6%, $p = 0.033$) and DFS (5-year DFS rate: 41.6% versus 25.0%, $p = 0.020$). No significant difference was observed when nN3 was compared with either the nN1 or nN2 subgroup ($p = 0.375$ and 0.759, respectively) (Figures 6*A*, *B*). The survival curves for OS and DFS showed distinct differences between the nN categories when pN2 patients were subdivided into different nN subgroups (5-year OS rate for nN1, nN2, and nN3 subgroup was 72.0%, 45.6%, and 19.4%, respectively, $p < 0.001$; 5-year DFS rate was 48.0%, 23.0%, and 3.9%, respectively, $p < 0.001$) (Figures 6*C*, *D*).

Each nN category was subdivided into pN1 and pN2 subgroups, and no significant difference in OS or DFS was observed between the pN1 and pN2 subgroups (Figures 7*A–F*).

In a Cox regression analysis, the nN category was identified as an independent prognostic factor for OS and DFS (versus nN3, the hazard ratios [HR] of nN0, nN1, and nN2 for OS were 0.123, 0.347, and 0.536 respectively, $p < 0.001$ for all of them; for DFS, the values were 0.088, 0.333, and 0.543, respectively, $p < 0.001$ for all of them) (Table 3). The HR of pN1 versus pN2 was not significant for OS (HR 0.729, $p = 0.081$) and was just narrowly significant for DFS (HR 0.760, $p = 0.041$) (Table 4). The HR of pN0 versus pN2 for OS and DFS were significant (0.222 and 0.173, respectively, $p < 0.001$ for both).

DISCUSSION

The TNM stage classification was developed to provide high specificity for patients with a similar prognosis and treatment options. As an essential component of this classification, nodal involvement was considered to be one of the

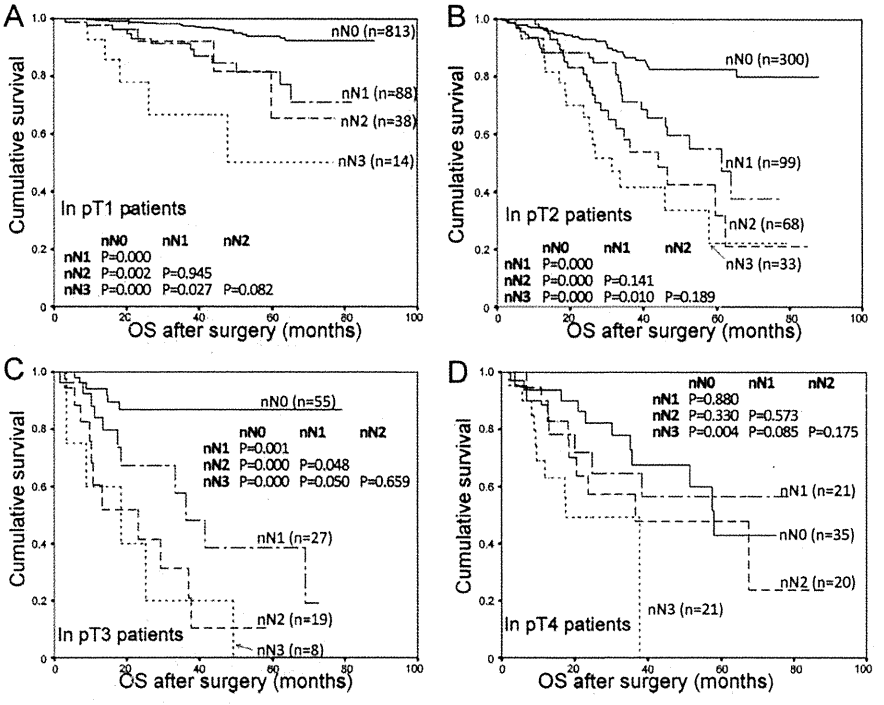

FIGURE 5. Overall survival (OS) curves according to nN category across each pT stage. *A*, OS curves according to nN category in pT1 patients. Five-year OS rate for nN0, nN1, nN2, and nN3 was 94.1%, 81.5%, 65.5%, and 50.1%, respectively. *B*, OS curves according to nN category in pT2 patients. Five-year OS rate for nN0, nN1, nN2, and nN3 was 82.6%, 55.0%, 31.9%, and 22.3%, respectively. *C*, OS curves according to nN category in pT3 patients. Five-year OS rate for nN0, nN1, nN2, and nN3 was 87.04%, 38.5%, 10.4% and 0%, respectively. *D*, OS curves according to nN category in pT3 patients. Five-year OS rate for nN0, nN1, nN2, and nN3 was 43.0%, 56.6%, 47.8%, and 0%, respectively.

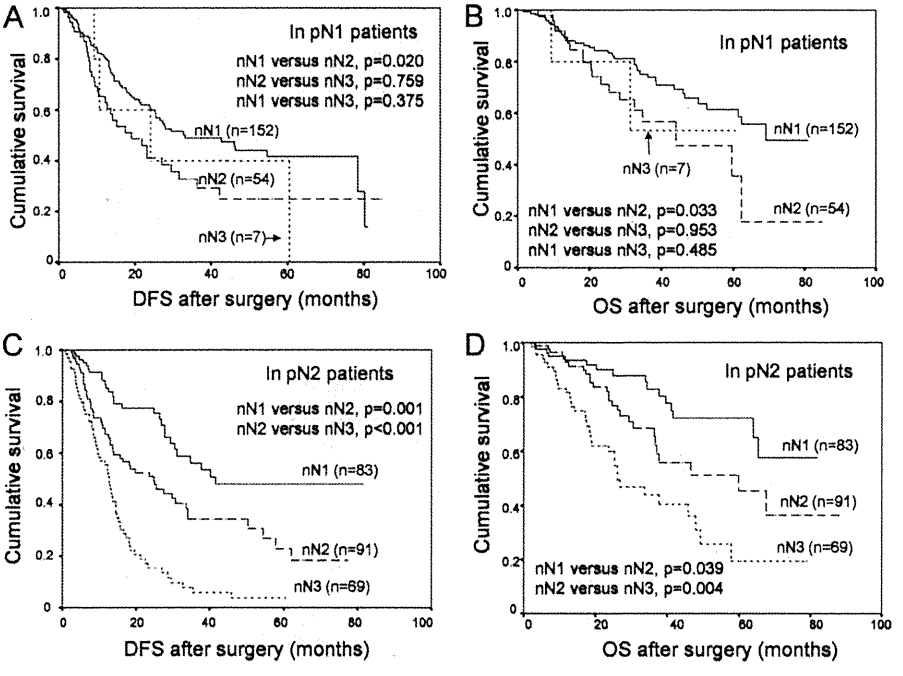

FIGURE 6. Disease-free survival (DFS) and overall survival (OS) according to nN subgroup when pN1 and pN2 patients were subdivided into different nN subgroups. *A*, DFS curves according to nN subgroup in pN1 patients. Five-year DFS rate for nN1, nN2, and nN3 subgroup was 41.6%, 25.0%, and 40%, respectively. *B*, OS curves according to nN subgroup in pN1 patients. Five-year OS rate for nN1, nN2, and nN3 subgroup was 61.5%, 35.6%, and 53.3%, respectively. *C*, DFS curves according to nN subgroup in pN2 patients. Five-year DFS rate for nN1, nN2, and nN3 subgroup was 48.0%, 23.0%, and 3.9%, respectively. *D*, OS curves according to nN subgroup in pN2 patients. Five-year OS rate for nN1, nN2, and nN3 subgroup was 72.0%, 45.6%, and 19.4%, respectively.

most important prognostic factors that influenced the survival of patients after surgery for primary NSCLC. The latest TNM staging system for lung cancer included notable changes in the T and M descriptors and the nodal map. However, the N descriptor remained the same as that in the sixth edition and depended solely on the anatomic extent of lymph node involvement.[1]

The location-based pN classification has some unsatisfactory aspects. The most important of these is the heterogeneity of pN1 and pN2 with regard to prognosis, which has been well documented, and subclassifications have been proposed.[2–13] In addition, differences among surgeons in the labeling of lymph node stations during surgery will always occur despite the introduction of a new nodal map. For

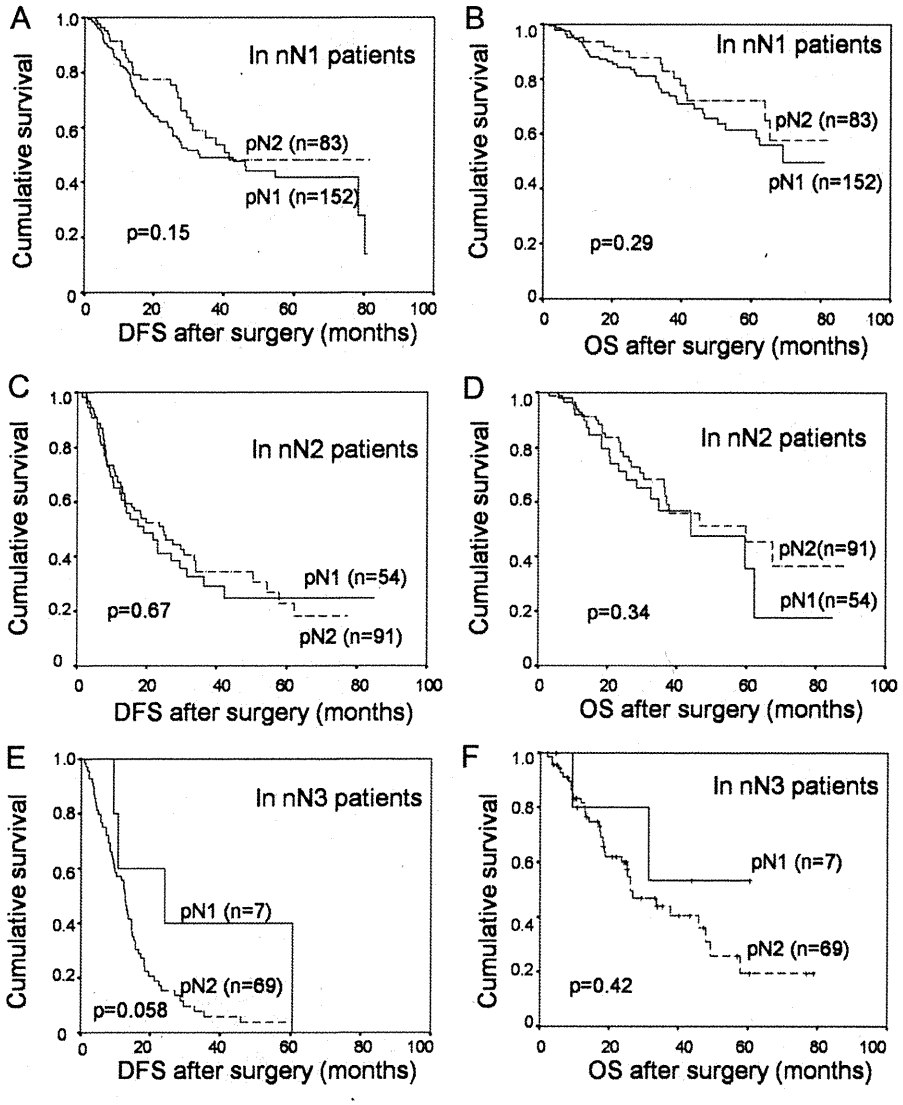

FIGURE 7. Disease-free survival (DFS) and overall survival (OS) according to pN subgroup when nN1, nN2, and nN3 patients were subdivided into pN1 and pN2 subgroups. *A*, DFS curves according to pN subgroup in nN1 patients. Five-year DFS rate for pN1 and pN2 subgroup was 41.6% and 48.0%, respectively. *B*, OS curves according to pN subgroup in nN1 patients. Five-year OS rate for pN1 and pN2 subgroup was 61.5% and 72.0%, respectively. *C*, DFS curves according to pN subgroup in nN2 patients. Five-year DFS rate for pN1 and pN2 subgroup was 25.5% and 23.0%, respectively. *D*, OS curves according to pN subgroup in nN2 patients. Five-year OS rate for pN1 and pN2 subgroup was 35.6% and 45.6%, respectively. *E*, DFS curves according to pN subgroup in nN3 patients. Five-year DFS rate for pN1 and pN2 subgroup was 40.0% and 3.9%, respectively. *F*, OS curves according to pN subgroup in nN3 patients. Five-year OS rate for pN1 and pN2 subgroup was 53.3% and 19.4%, respectively.

example, if a lymph node is located on the border between stations 4 and 10 during an operation, the same lymph node may be labeled as either N1 or N2 by different surgeons. Therefore, it is necessary to develop a refined nodal category that is simple and easy to use to provide more accurate prognostic stratifications.

In the TNM classification for some other tumors, such as gastric, breast, and colorectal cancer, the number of positive lymph nodes has been considered in the definition of pN categories.[14] In recent years, the association between the number of metastatic lymph nodes and the prognosis in resected NSCLC has also been explored. Lee et al.[15] demonstrated a stepwise deterioration with an increase in the number of positive nodes. Excellent agreement was observed between the pN and nN categories. In another study by Fukui et al.,[9] the number category defined in their study was shown to be an independent prognostic factor that could be used to stratify pN2 patients into homogenous subgroups. However, neither of these studies determined whether the pN stage classification or the nN category is the better prognostic factor. In this study, we retrospectively explored the association between the number of metastatic lymph nodes and the prognosis in 1659 resected NSCLC patients and compared the results with the classic pN stage.

In our series, although there was a significant difference in DFS between pN1 and pN2, the OS curves for the two categories were close to each other, and no significant difference was observed ($p = 0.245$). A multivariate analysis demonstrated similar results. These results implied that the pN classification had poor discriminative ability with regard to the prognosis in patients with lymph node metastasis and were very different from the report by Fukui et al.[9] and other prior reports.[11,19] While the reason for this difference is unclear, it is notable that the 5-year OS rate of pN2 patients in our study was 47.8%, which was much higher than that in prior reports[9,11] and close to that for pN1. This result might be due to the fact that all the pN2 patients who underwent surgery in this study were mN2 disease (N2-positive based

TABLE 3. Multivariate Analysis of OS and DFS Including nN Category

	OS			DFS		
Variable	Hazard Ratio	95% CI	*p*	Hazard Ratio	95% CI	*p*
Gender	0.621	0.449–0.859	0.004	—	—	—
Age	1.014	0.998–1.029	0.083	1.013	1.002–1.024	0.025
Surgical procedure (reference: wedge resection/segmentectomy)						
Lobectomy	—	—	—	0.530	0.320–0.877	0.013
Bilobectomy	—	—	—	0.465	0.244–0.887	0.020
Pneumonectomy	—	—	—	0.609	0.328–1.131	0.116
Surgical margin	1.838	1.251–2.699	0.002	1.585	1.153–2.179	0.005
Histological type (reference: adenocarcinoma)						
Squamous cell carcinoma	1.200	0.852–1.691	0.297	0.755	0.569–1.002	0.052
Large cell carcinoma	1.625	0.881–2.997	0.120	1.759	1.093–2.832	0.020
Adenosquamous carcinoma	3.386	1.616–7.097	0.001	2.528	1.271–5.029	0.008
pT stage (reference: pT1)						
pT2	2.676	1.841–3.890	<0.001	2.257	1.742–2.923	<0.001
pT3	4.571	2.842–7.350	<0.001	3.966	2.745–5.732	<0.001
pT4	4.556	2.861–7.257	<0.001	4.032	2.888–5.627	<0.001
nN category (reference: nN3)						
nN0	0.123	0.080–0.191	<0.001	0.088	0.063–0.125	<0.001
nN1	0.347	0.222–0.542	<0.001	0.333	0.237–0.468	<0.001
nN2	0.536	0.343–0.836	<0.001	0.543	0.389–0.759	<0.001

OS, overall survival; DFS, disease-free survival; nN, number of metastatic lymph nodes; CI, confidence interval.

TABLE 4. Multivariate Analysis of OS and DFS Including pN Stage

	OS			DFS		
Variable	Hazard Ratio	95% CI	*p*	Hazard Ratio	95% CI	*p*
Gender	0.673	0.486–0.932	0.017	—	—	—
Age	1.013	0.998–1.029	0.092	1.012	1.001–1.024	0.030
Surgical procedure (reference: wedge resection/segmentectomy)						
Lobectomy	—	—	—	0.548	0.331–0.905	0.019
Bilobectomy	—	—	—	0.557	0.294–1.056	0.073
Pneumonectomy	—	—	—	0.754	0.409–1.388	0.364
Surgical margin	2.141	1.470–3.118	<0.001	1.858	1.363–2.533	<0.001
Histological type (reference: adenocarcinoma)						
Squamous cell carcinoma	1.148	0.815–1.617	0.429	0.700	0.529–0.927	0.013
Large cell carcinoma	1.620	0.874–3.003	0.126	1.808	1.123–2.910	0.015
Adenosquamous carcinoma	3.609	1.725–7.547	0.001	2.498	1.259–4.954	0.009
pT stage (reference: pT1)						
pT2	2.880	1.989–4.171	<0.001	2.359	1.824–3.050	<0.001
pT3	4.529	2.811–7.298	<0.001	3.769	2.609–5.444	<0.001
pT4	4.714	2.962–7.503	<0.001	4.175	2.994–5.821	<0.001
pN stage (reference: pN2)						
pN0	0.222	0.156–0.315	<0.001	0.173	0.133–0.226	<0.001
pN1	0.729	0.512–1.040	0.081	0.760	0.583–0.989	0.041

OS, overall survival; DFS, disease-free survival; CI, confidence interval.

solely on a postoperative pathologic examination), and the exclusion criteria of cN2 disease was much stricter than that set by Fukui et al., who excluded only bulky N2 disease with extranodal invasion,[9] which resulted in a higher percentage and lower 5-year survival of pN2 population in their series than in ours (versus ours: 22% versus 14.6% and 40% versus

47.8%, respectively). In addition, refinement of the preoperative work-up to screen cN2 disease and distant metastasis also contributed to the higher OS rate of pN2.

We defined four nN categories according to the number of nodes with metastasis and examined the respective survival curves. All the patients were stratified into four prognostically distinct groups by the nN classification. When we tried to validate the results across each pT stage, a clear tendency of deterioration of OS from nN0 to nN3 in the same pT stage can be observed, and the curves were split in pT1 and pT2 stage. The results indicated that nN category was a prognostic factor even in the same pT stage. In the higher pT stage of pT3 and pT4, however, the curves were not apart from each other. In addition to the small number of the cases, the other reason may be that the prognosis of the higher pT stage was always poor regardless of the number of metastatic lymph nodes, so the prognostic effect of nN category was not well demonstrated in these populations.

In a multivariate analysis, the nN category was shown to be an independent prognostic factor for both OS and DFS. Furthermore, the nN category could be used to subdivide pN1 and pN2 patients into two (nN1 and nN2) and three (nN1, nN2 and nN3) prognostically distinct subgroups, respectively. These results showed that the nN category has a powerful discriminative ability with respect to the prognosis and that the pN1 and pN2 categories are prognostically heterogeneous. However, the survival of the nN3 subgroup in pN1 patients was not significantly different from that of the nN1 and nN2 subgroups. It seems that the nN classification does not have as strong a discriminative ability in pN1 as in pN2 patients. It is difficult to explain this finding; however, it may be, in part, due to the small size of the nN3 subgroup ($n = 7$) in pN1 patients. Another possible explanation is that lymph node fragments were removed. During the operation, some of the N1 lymph nodes were most likely removed in fragments instead of intact because of adhesion to the bronchus and lung tissues. Each fragment may have been counted as a single node during the pathologic examination. Thus, the true number of metastatic lymph nodes may have been overestimated, and this would bias the results toward null. In contrast, most of the mediastinal lymph nodes (N2) were removed en bloc with the adjacent soft tissue, and fewer fragments than N1 nodes were produced. All these factors may have contributed to the observed results.

When we subdivided the nN category into pN1 and pN2 subgroups, no significant survival difference was observed between the two subgroups. This indicated that, for metastasis in the same number of lymph nodes, the anatomic location of the positive node (N1 or N2) is not important for postoperative survival. We tend to agree with the opinion that the overall disease burden, rather than the anatomic location of lymph node involvement, has the most important influence on prognosis.[11] Based on the finding in this study that the pN2 stage was accompanied by more lymph node metastasis than the pN1 stage, we postulate that even the slightly higher DFS rate in pN1 than pN2 was attributed to the smaller number of metastatic lymph nodes.

Despite the benefits of nN category for predicting survival, it also has some limitations. As we discussed above, some lymph nodes were inevitably removed in fragments, especially in the N1 region, which could lead to an overestimation of the number of metastatic nodes. Such an overestimation would bias the results of this study toward null, and the true association between the nN category and survival may be stronger than what we observed. However, when the nN category is used clinically as a prognostic tool, the survival risk of patients may be overestimated because of the presence of nodal fragments. To avoid the overestimation, the surgeon should remove the lymph nodes en bloc with the adjacent soft tissue to avoid fragments. If the fragment is inevitable, it is necessary for the operator to put the fragments from one single lymph node into a same bottle and label it definitely.

Second, a sufficient number of retrieved lymph nodes is essential to evaluate the true number of metastatic nodes. In gastric cancer, at least 15 removed lymph nodes are required to assure the reliability of the pN classification.[20] In lung cancer, there have long been controversies regarding the extent of lymphadenectomy.[18,21–23] Some reports have suggested that the optimal number of removed lymph nodes is 11 to 16 to accurately assess stage I lung cancer.[24,25] In the study by Lee et al.,[15] the removal of 11 nodes was set as a threshold for inclusion in their study. In our study, we did not set a threshold. We performed selective lymph node dissection based on the lobe-specific patterns of nodal metastasis for all but the high-risk patients. We think that the number of metastatic lymph nodes should be stable as long as less dissection is based on the idea of the lobe-specific nodal metastasis.[16,17,21,22]

Third, it is difficult to accurately assess the number of metastatic lymph nodes both preoperatively and in nonsurgical patients by CT scan or other methods currently used. Although PET scan can discriminate some metastatic lymph nodes, this is not sufficient to determine the nN category. Therefore, the nN category will contribute less to determine the optimal treatment before surgery. New methods that are capable of identifying each metastatic lymph node for nonsurgical patients will need to be developed.

Finally, the optimal category definition for the number of metastatic lymph nodes needs to be further explored. In this study and previous studies by Lee et al.[15] and Fukui et al.,[9] four categories were defined, and the patients without lymph node metastasis were grouped into a single category. However, for patients with metastatic lymph nodes, the categories had different definitions. Both the other two studies showed the prognostic significance of the number of metastatic lymph nodes based on their category definitions. Because the data are from different institutes, it is difficult to discuss which category definition is the best. Further studies are needed.

In summary, our results demonstrated that the location-based pN stage classification had a poor discriminative ability with regard to the prognosis in resected NSCLC, and patients in pN1 and pN2 are prognostically heterogeneous. Despite the limitations, the nN category as defined in this study is a better prognostic determinant than the location-based pN

stage. The overall disease burden, rather than the anatomic location of lymph node involvement, may have the most important influence on the prognosis. Furthermore, the number of metastatic lymph nodes is a more objective measure than their location, because errors could be made in determining the location of metastatic nodes. Therefore, we believe that the number of metastatic lymph nodes should be considered for the nodal stage classification in the future.

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Frequent *ALK* rearrangement and TTF-1/p63 co-expression in lung adenocarcinoma with signet-ring cell component

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ABSTRACT

Primary adenocarcinoma with signet-ring cell component (Ad-SRCC) of the lung has been well characterized clinicopathologically and histologically, but their genetics has rarely been investigated. A recent report suggesting an association between Ad-SRCC and *EML4-ALK* fusion prompted us to undertake a histological, immunohistochemical, and molecular analysis of 10 cases of primary Ad-SRCC identified out of 699 lung adenocarcinomas (1.4%). Most of the Ad-SRCCs showed characteristic architectural as well as cytological features including cohesive clustering of signet-ring cells, a solid/acinar growth pattern, and alveolar filling at the tumor periphery. Diffuse co-expression of TTF-1 and p63 was observed in half of the Ad-SRCCs, and this immunoprofile has not been recognized previously. Four Ad-SRCCs (40%) harbored *ALK* translocations detected by reverse-transcriptase polymerase chain reaction, fluorescence in situ hybridization, and immunohistochemistry. One new *EML4-ALK* fusion variant was identified. One *ALK*-rearranged tumor showed focal squamous differentiation. None of the present Ad-SRCCs had *EGFR* or *KRAS* mutations, regardless of *ALK* status. This study successfully utilized tumor histology alone to identify a subset of adenocarcinomas showing a high rate of *ALK* translocation. The characteristic histology, immunoprofile, frequent *ALK* translocation, and total lack of *EGFR* or *KRAS* mutations, may suggest that Ad-SRCC forms a histologically/molecularly coherent subgroup of adenocarcinoma.

1. Introduction

Lung cancer is the leading cause of cancer death both in men and women worldwide. Surgery, chemotherapy, and radiation therapy are the standard therapeutic modalities [1], and the treatment of lung cancer has conventionally been dictated by histological classification and tumor stage [1,2]. In recent years, the classification of lung cancer has become refined by molecular genetic data, and this trend has important therapeutic implications, helping to guide clinicians to the optimal treatment for individual patients [3,4].

In 2007, Soda et al. [5] discovered a novel transforming fusion gene joining the echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (*ALK*) genes in a subset of non-small-cell lung carcinoma (NSCLC). The *EML4-ALK* fusion gene is formed by a small inversion within the short arm of chromosome 2, and the encoded protein, a chimera comprising the N-terminal part of EML4 and the intracellular catalytic domain of ALK, is expressed constitutively and dimerized without ligand stimulation [5]. The presence of the *EML4-ALK* fusion in NSCLCs was subsequently confirmed by other investigators worldwide [6–16]. A number of fusion variants have been identified to date, and another rare fusion partner for *ALK* is *KIF5B* [15]. Since ALK is a tyrosine kinase receptor, this subtype of NSCLC is expected to be a good candidate for treatment with small-molecule ALK tyrosine kinase inhibitors [2,17]. Several studies have already confirmed that such drugs induce growth cessation of *ALK*-translocation-positive NSCLC in vitro [5,10] and in xenografts [10,18]. A preliminary phase I study of one of such drugs yielded promising results in a cohort of patients with *ALK*-rearranged NSCLCs [19].

Signet-ring cell component in primary adenocarcinoma of the lung has been recognized for more than two decades [20]. The clinicopathological features of adenocarcinoma with signet-ring cell component (Ad-SRCC) have been well described in the literature [21,22], and its histological features repeatedly documented [21,23–25]. However, the genetic background of this subtype of adenocarcinoma has not been investigated in detail [24,26], and

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there has been a paucity of comprehensive analyses of Ad-SRCCs covering both phenotypic and molecular genetic aspects. Recently, Rodig et al. [11] identified frequent signet-ring cell populations in 20 adenocarcinomas that carried *ALK*-rearrangement by fluorescence in situ hybridization (FISH) analysis. Prompted by this report, we examined 10 consecutive cases of surgically resected Ad-SRCCs of the lung. Along with a detailed histological study and standard *EGFR* and *KRAS* mutation assays, we investigated the *ALK* status of these tumors using multiplex reverse-transcription polymerase chain reaction (RT-PCR), FISH, and immunohistochemistry (IHC).

2. Materials and methods

2.1. Case selection

We reviewed 699 consecutive primary adenocarcinomas of the lung resected at the National Cancer Center (NCC), Tokyo, in 2004 and 2005, and retrieved cases of Ad-SRCC. The possibility of metastasis from other sites was clinically/radiologically excluded. The diagnosis of Ad-SRCC was based on the presence of signet-ring cells, which have marginally located crescentic nuclei and abundant intracytoplasmic mucin, the latter being highlighted dark blue by alcian blue- periodic acid-Schiff (AB-PAS) staining. We accepted tumors as Ad-SRCCs when unequivocal signet-ring cells accounted for ≥5% of the total tumor cells. This cutoff is chosen because there is no universally agreed upon threshold, and because routine histological examination can readily identify signet-ring cells when they occupy at least 5% of the tumor cells based on our experience and that of others [27]. The signet-ring cell percentage was calculated after reviewing all the available slides, which represented the entire lesion in small-sized tumors (3.0 cm or less), or the product of adequate sampling in larger tumors. Mucinous bronchioloalveolar carcinoma (BAC), colloid carcinoma, and solid adenocarcinoma with mucin production may contain variably shaped mucus cells [23,28], but they generally lack signet-ring cells ≥5% and are therefore differentiated from Ad-SRCC. Each adenocarcinoma was estimated for tumor size, percentage of signet-ring cells among total tumor cells, nuclear grade (low, intermediate, or high), TNM stage [29], and predominant growth pattern as defined by the World Health Organization (WHO) [1]. Clinical information for each case was collected by reviewing the medical records. This study was approved by the Institutional Review Board of NCC.

2.2. IHC

Four-micrometer-thick sections were deparaffinized. Heat-induced epitope retrieval was performed with 1.0-mmol/L citrate buffer (pH 6.0) for ALK protein and TTF-1, and with TRS9 (DAKO, Carpinteria, CA) for p63. The slides were treated with 3% hydrogen peroxide for 20 min. The slides were then incubated with primary antibodies against ALK protein (1:40, ALK1, DAKO), p63 (1:400, 4A4, DAKO), and TTF-1 (1:100, 8G7G3/1, DAKO) for 1 h at room temperature. Immunoreactions were detected using the Envison-plus system (DAKO) for p63 and TTF-1, and CSAII (DAKO) for ALK protein. The reactions were visualized with 3,3′-diaminobenzidine. Appropriate positive and negative controls were used. Only the nuclear stain was deemed positive for TTF-1 and p63, and the extent of staining was graded as 0 (0–10%), 1+ (>10–25%), 2+ (>25–50%), and 3+ (>50%). Strong diffuse granular cytoplasmic staining was regarded as positive for ALK.

2.3. FISH

FISH was performed on formalin-fixed, paraffin-embedded tumor tissues using a break-apart probe for the *ALK* gene (Vysis LSI ALK Dual Color, break-apart rearrangement probe; Abbott Molecular) in accordance with the manufacturer's instructions. Positive rearrangement was defined as a splitting apart of the fluorescence probes flanking the *ALK* locus. In addition, as recently shown by others in abstract form [30], loss of 5′ locus (green signal) of split-apart *ALK* was considered equivalent to the *ALK* rearrangement, likely reflecting the loss of non-functioning *ALK-EML4* fusion product. Three experienced observers independently assessed the slides. Adjacent uninvolved lung tissue was used as negative control. Decisions regarding positivity and negativity required unanimous agreement among three observers, and cases for which opinions were divided were designated indeterminate for *ALK* rearrangement.

Table 1
Primers used for RT-PCR and sequencing.

For detection of *EML4-ALK* fusion	
EA-F1	5′ GTGCAGTGTTTAGCATTCTTGGGG 3′
EA-F2	5′ AGCTACATCACACACCTTGACTGG 3′
EA-F3	5′ TACCAGTGCTGTCTCAATTGCAGG 3′
EA-F4	5′ GCTTTCCCCGCAAGATGGACGG 3′
ALK-R	5′ TCTTGCCAGCAAAGCAGTAGTTGG 3′
For detection of *KIF5B-ALK* fusion	
KA-F1	5′ CAGCTGAGAGAGTGAAAGCTTTGG 3′
KA-F2	5′ GACAGTTGGAGGAATCTGTCGATG 3′
KA-F3	5′ ATCCTGCGGAACACTATTCAGTGG 3′
KA-F4	5′ TCAAGCACATCTCAAGAGCAAGTG 3′
ALK-R	5′ TCTTGCCAGCAAAGCAGTAGTTGG 3′
For detection of *EGFR* mutation	
EGFR-RTF1	5′ CCTCTTACACCCAGTGGAGAAGC 3′
EGFR-RTR1	5′ CAGTTGAGCAGGTACTGGGAGCC 3′
EGFR-RTF2	5′ TCCTGGACTATGTCCGGGAACAC 3′
EGFR-RTR2	5′ AGGTCATCAACTCCCAAACGGTC 3′
For detection of *KRAS* mutation	
KRAS-RTF1	5′ AGAGAGGCCTGCTGAAAATGACTG 3′
KRAS-RTR1	5′ CCATAGGTACATCTTCAGAGTCC 3′

2.4. RT-PCR and sequencing for ALK fusions

Frozen tumor tissues were powdered by CP02 (Covaris, Woburn, MA) and sonicated using a Covaris S2 (Covaris). Total RNA was extracted using a mirVana RNA Isolation Kit (Ambion, Foster City, CA). cDNA was synthesized with MMTV reverse transcriptase (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Switzerland). To amplify *ALK* fusion genes, a mixture of primers covering potential breakpoints of fusion transcripts (*EML4-ALK* and *KIF5B-ALK*, respectively) were used as reported previously (the sequences of the primers used are listed in Table 1) [7,15]. The multiplex PCR conditions were 95 °C for 60 s, followed by 50 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 60 s. The PCR products were electrophoresed, and potential fusion transcripts were purified and sequenced with an ABI 3130 Sequencer using PCR primers (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). In addition, the PCR products were subcloned into a TA-cloning vector (Invitrogen, Carlsbad, CA) and sequenced using M13 primers.

2.5. EGFR and KRAS mutation analysis

In cases 1–9, partial cDNAs of the *EGFR* (codon 700–909) and *KRAS* (codon 1–108) genes covering potential mutation hotspots were amplified by RT-PCR and sequenced as described above. The primer sequences are listed in Table 1. Case 10, for which frozen material was not available, was studied by high-resolution melting analysis for the common *EGFR* (L858R mutation and exon 19 deletion) and *KRAS* (codons 12 and 13) alterations, as performed routinely at our institution [31].

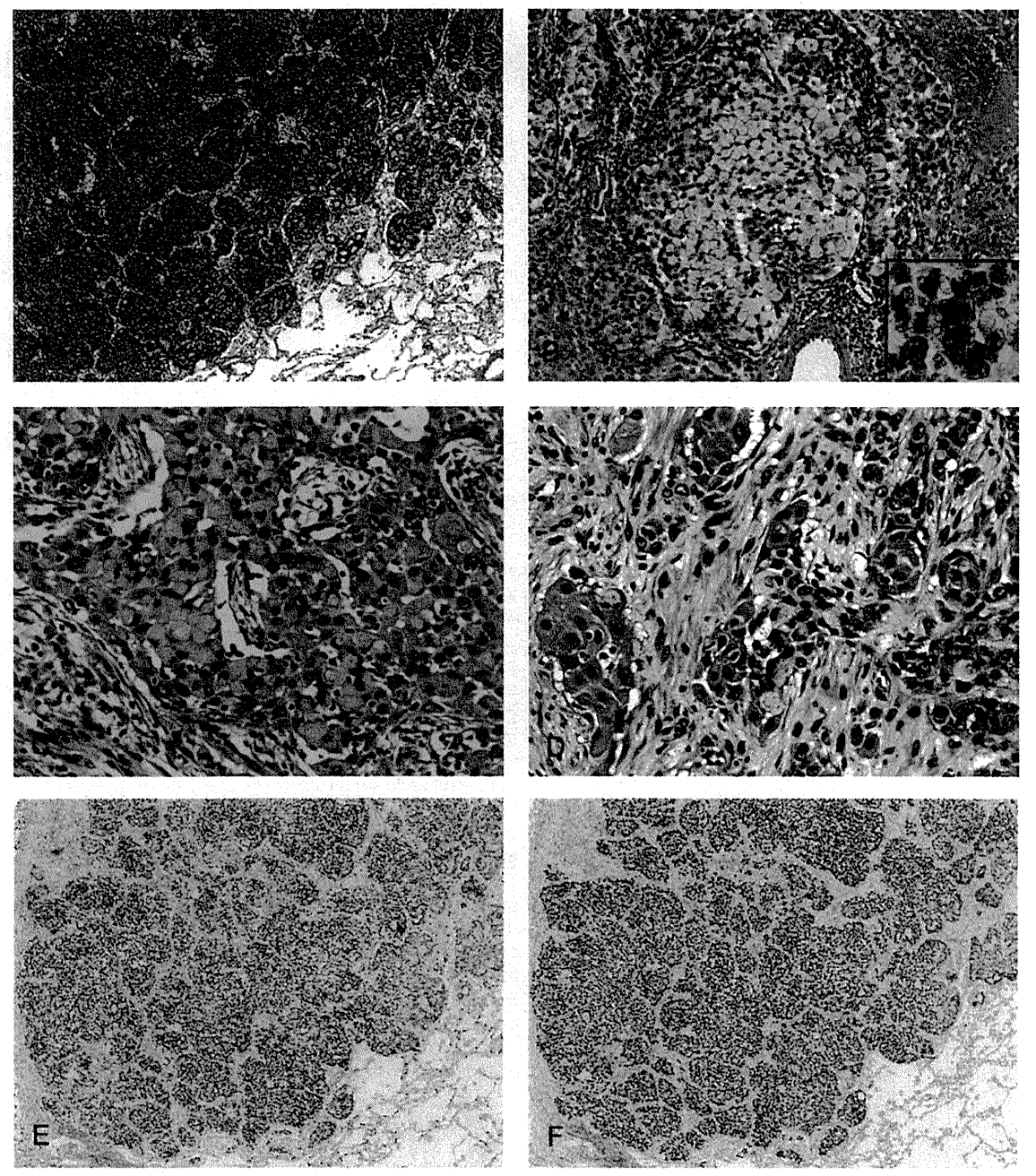

Fig. 1. Histological features of Ad-SRCCs of the lung showing solid/acinar growth with an alveolar filling pattern at the lesion periphery (A, H&E, case 1). Signet-ring cells were present at the center of a large tumor cell nest. Notice characteristic cohesive clustering of signet-ring cells and relatively monomorphic nuclei. Intracytoplasmic mucin in signet-ring cells was highlighted dark blue by alcian blue-periodic acid-Schiff (AB-PAS) staining, shown in the inset (B, H&E, case 6; inset, AB-PAS). Clusters of signet-ring cells in smaller nests (C, H&E, case 7). One tumor harboring the *EML4-ALK* fusion gene showed focal squamous differentiation (D, H&E, case 10). Fifty percent of Ad-SRCCs diffusely co-expressed TTF-1 (E, TTF-1 immunostain, case 1) and p63 (F, p63 immunostain, case 1).

3. Results

3.1. Clinicopathological features

Ten (1.4%) cases of lung Ad-SRCC were identified out of 699 consecutive primary adenocarcinomas resected at NCC Tokyo (2004–2005). The pertinent clinicopathological data are summarized in Table 2, along with the immunohistochemical and genetic results. The patients were five men and five women with a mean age of 58 (range, 33–78) years. They were slightly younger than 699 primary lung adenocarcinoma patients treated during the study period (mean age 63.4 years old, $p = 0.038$). There were five non-smokers, two light smokers (<20 pack-years), and three heavy smokers (≥20 pack-years). The tumors all appeared well circumscribed on gross examination and measured 2.7 cm in diameter on average (range, 1.0–5.0 cm). Five tumors were found at stage I, one was at stage II, and three were at stage III. Histologically, all the tumors contained, by definition, at least 5% signet-ring cells. AB-PAS staining highlighted mucin as dark blue spherules in all cases (Fig. 1B, inset). The ratio of signet-ring cells relative to the total tumor cells varied with an average of 13% (range, 5–30%). In all cases, the signet-ring cells formed tight or loose cohesive

Table 2
Clinicopathological findings, and *EGFR*, *KRAS*, and *ALK* status of the present 10 Ad-SRCCs of the lung.

Case #	Age	Sex	SI	Size (cm)	Stage	Follow-up, months	WHO predominant pattern	SRC%	Nuclear grade	TTF-1 IHC	p63 IHC	*ALK* RT-PCR	*ALK* FISH	ALK IHC	*EGFR*	*KRAS*
1	34	F	14	1.8	IA	NED, 60	Solid	20	Low	3+	3+	+	+[a]	+	WT	WT
2	60	M	29	1.8	IA	NED, 64	Acinar	5	Low	3+	3+	–	NA	–	WT	WT
3	56	F	0	4.5	IIIA	DOD, 32	Solid	5	Low	3+	1+	–	–	–	WT	WT
4	63	M	30	5.0	IIIA	AWD, 3	Acinar	5	Intermediate	3+	1+	–	Ind	–	WT	WT
5	78	M	10	3.0	IA	AWD, 53	Solid	5	Intermediate	0	0	–	–	–	WT	WT
6	63	F	0	3.0	IIIB	NED, 50	Solid	20	Low	3+	3+	–	–	–	WT	WT
7	68	M	30	2.5	IIA	NED, 51	Solid	5	Intermediate	2+	2+	–	+	–	WT	WT
8	77	F	0	2.5	IA	NED, 48	Papillary	15	Low	3+	2+	+	Ind	+	WT	WT
9	51	M	0	1.0	IIIA	NED, 47	Solid	20	Low	3+	3+	+	+	+	WT	WT
10	33	F	0	1.4	IB	NED, 62	Solid	30	Intermediate	3+	3+	NA	+[a]	+	WT	WT

F, female; M, male; SI, smoking index (number of packs of cigarettes per day × years); NED, no evidence of disease; DOD, died of disease; AWD, alive with disease; SRC%, percentage of signet-ring cells; NA, not available; Ind, indeterminate; WT, wild type.
[a] Cases showing a single orange signal and one fused signal on FISH.

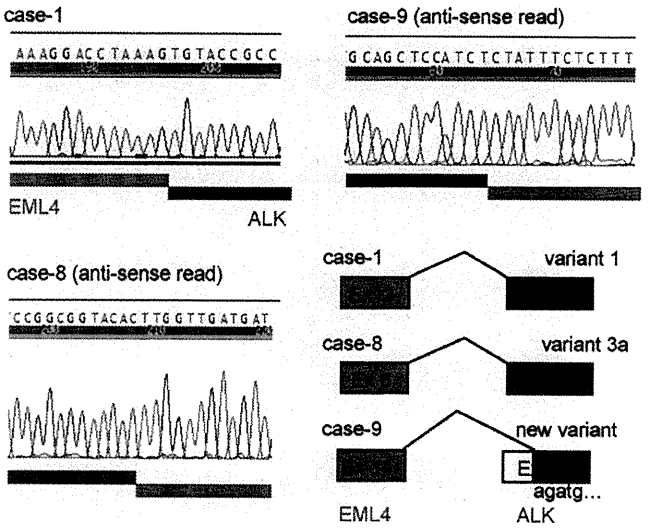

Fig. 2. RT-PCR and sequence analysis identified the presence of *EML4-ALK* fusion transcripts in three cases (cases 1, 8, and 9) of Ad-SRCC.

clusters within solid nests of tumor cells (Fig. 1B and C), typically appearing in the central portion of nests. The tumor cell nests filled and expanded the alveolar spaces at the lesional periphery in nine cases (Fig. 1A). The tumor cell nuclei were relatively monomorphic, except for focal areas of pleomorphism in case 4, and showed low- (six cases) to intermediate-grade (four cases) atypism. The predominant WHO growth pattern was solid in seven cases, acinar in two, and papillary in one. Signet-ring cells were associated with a solid pattern in eight cases, an acinar pattern in one, and both acinar and solid patterns in one. Lepidic growth was absent in all the cases except for case 6. One tumor (case 10) showed focal squamous differentiation (Fig. 1D). Extracellular mucin was minimal in all cases. Nine Ad-SRCCs were diffusely immunopositive for TTF-1 (Fig. 1E). Nine tumors were also immunoreactive for p63, and five of them showed diffuse (>50%) labeling with strong intensity (Fig. 1F). In seven cases, the foci of signet-ring cells within the tumor co-expressed TTF-1 and p63 (cases 1, 2, 6–10). Follow-up information was available for all of the patients. Seven patients were alive and well without recurrence after a mean follow-up period of 55 months (range, 47–64 months), two patients were alive with distant recurrence at 3 and 53 months, respectively. The remaining patient died of the disease 32 months after surgery.

3.2. *ALK* analysis (RT-PCR, FISH, and IHC)

The expected PCR products of the *EML4-ALK* fusion gene were observed in three out of nine tested cases, and none of the *KIF5B-ALK* fusion transcripts was amplified. The sequence of each PCR product revealed that these three cases had different fusion transcripts (Fig. 2). The detected transcripts were variant 1 in case 1 and variant 3a in case 8. Case 9 harbored a new breakpoint connecting *EML4* exon 14 and the 12-amino-acids-deleted *ALK* exon 20.

Among the three RT-PCR-proven ALK-translocated cases, unanimous agreement on the positive *ALK* rearrangement was obtained for two tumors by FISH (Fig. 3). The remaining case (case 8) was designated as indeterminate for *ALK* rearrangement, and it showed more than 2 pairs of signals in the vast majority of the tumor cells, the significance of which finding was unclear. Among the six PCR-negative cases, FISH was unsuccessful in one case, three were designated as negative, one was designated as indeterminate, and one was designated as positive for *ALK* rearrangement. The latter case (case 7) showed a small number of tumor cells exhibiting wider split signals than expected for *EML4-ALK* fusion. Case 10, whose

Fig. 3. FISH study using an *ALK* break-apart probe showed *ALK* rearrangement (splitting of green and orange signals) in an Ad-SRCC (case 9).

fresh tissue was not available for RT-PCR analysis, was shown to be positive for *ALK* rearrangement by FISH. In cases 1 and 10, more than 50% of the counted 100 tumor cells demonstrated loss of 5′ locus of split-apart *ALK*, showing one fused signal and one orange signal per cell.

The results of IHC were in accordance with those of PCR. All of the three PCR-positive tumors were strongly reactive for ALK antibody (Fig. 4), and all of the six PCR-negative tumors were non-reactive for this marker. Case 10, in which *ALK* rearrangement was detected by FISH, also showed strong labeling for ALK antibody.

3.3. *EGFR and KRAS mutation analyses*

No mutation of the *EGFR* or *KRAS* gene was detected by sequencing in the nine studied cases. Case 10 was also negative for *EGFR* or *KRAS* mutation by high-resolution mutation analysis.

4. Discussion

The clinical and histological findings in the present series were generally in accord with prior reports. The incidence of Ad-SRCCs in this series was 1.4%, in keeping with the rarity (0.14–1.9%) reported by other authors [21–23,26]. Younger age at onset and light tobacco exposure in the present series also concurred with the findings of other studies [21–24,32]. Unlike others [22,23,27], however, the majority of the tumors in this series had a lower stage (I or II) at presentation, and their prognoses were accordingly fair, but these findings likely reflect that all of our cases were surgically resected at the institution which treats many small-sized lung tumors. Besides their defining cytological attribute, Ad-SRCCs showed characteristic architectural profiles as described previously [21,24,27], including solid nests, cohesive clustering of signet-ring cells, and alveolar filling patterns at the lesional periphery [21,23,33]. The cohesiveness of signet-ring cells is somewhat at variance with most homonymous tumors in the stomach or breast, which often infiltrate diffusely as isolated cells. The nuclei of the Ad-SRCCs in this series were uniform and of low to intermediate grade without frank anaplasia in most cases, though the nuclear features of Ad-SRCCs have not been fully analyzed in the past [23].

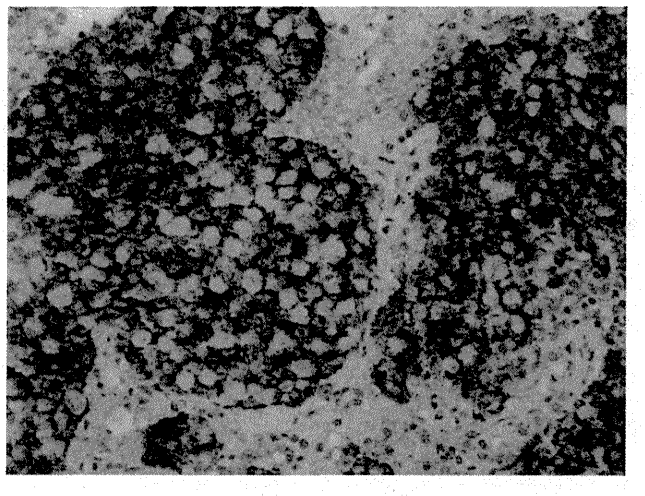

Fig. 4. ALK protein is immunohistochemically positive in an Ad-SRCC (ALK immunostain, case 1).

Immunohistochemically, most of our Ad-SRCCs were positive for TTF-1, as expected [23,26]. Interestingly, most tumors also labeled for p63, and half were stained in diffuse strong manner by this antibody. The immunoreactivity of p63 in lung adenocarcinomas is considered to be uncommon [34,35], and a recent report in abstract form [36] showed that significant co-expression of TTF-1 and p63 occurred in only 5.5% of adenocarcinomas of the lung in general. This previously unrecognized peculiar immunoprofile of Ad-SRCC may indicate that this tumor subtype might arise from a specific cell of origin, different from most lung adenocarcinomas. Although diffuse strong p63 positivity is often used as a marker of squamous cell carcinoma in diagnostic pathology, Ad-SRCC seems a major pitfall to this practice. Careful attention to the focal signet-ring cell element and TTF-1 staining should lead to the correct diagnosis.

Since ALK analysis was partly complicated by the technical difficulty of FISH (see below), we required evidence of *ALK* alteration on the basis of at least two different modalities for the diagnosis of *ALK*-translocated cancer. Four of the 10 cases (40%) of lung Ad-SRCC were thus regarded as positive for *ALK* rearrangements (cases 1, 8, 9, and 10). This result is in accord with Rodig et al. [11], who found that 14 of 47 (30%) Ad-SRCCs showed *ALK* rearrangement by FISH. *ALK*-rearranged tumors in this study had a significantly more proportion of signet-ring cell components than *ALK* wild-type tumors (mean 44% vs. 10%; $p = 0.0058$), and this trend was also in agreement with the previous report [11]. Although it was suspected that the presence of signet-ring cell in *ALK*-rearranged tumors might be a regionally/ethnically limited phenomenon [11], we showed that it is rather a universal finding also evident in non-Western patients.

Because the *EML4-ALK* fusion in lung cancer is rare (3.8%) [17] in unselected populations, and because the currently accepted methods for detecting this chimeric gene are relatively expensive and labor-intensive, a practical concentration strategy is needed for effectively preselecting a subgroup of patients whose tumors are more likely to be positive for *ALK* translocation [2]. The present study used histological criteria alone, i.e. those of Ad-SRCC, to successfully extract a subset of adenocarcinomas carrying *ALK* translocation in as many as 40% of the cases. Other clinical (e.g., younger age and minimal tobacco exposure) and histological (e.g., solid or acinar pattern) features known to be associated with *ALK*-rearranged tumors [8,12] may also be used in combination with the signet-ring cells in order to enhance the detection rate. Rodig et al. [11] indeed noted that as many as 50% of their tumors showing a combination of solid growth and the presence of >10% signet-ring cells harbored *ALK* rearrangement.

The minor discordance of *ALK* status among the modalities used in this study resulted primarily from FISH analysis, whereas the results of RT-PCR and IHC were completely concordant. Interpretation of FISH results for *EML4-ALK*-positive lung cancer is known to

be technically difficult [2,11]. Because both the *EML4* and *ALK* genes are located close to each other on the same chromosome arm, their fusion yields a split signal separated by only a short distance. Consequently, identifying a split is not as straightforward as in other translocation-associated tumors, and the criteria for recognition of positive split signals may vary among observers. Such variability may well have contributed to the present FISH results, for which opinions on two cases conflicted among different observers. One case (case 7) was interpreted as FISH-positive, as opposed to the results of RT-PCR and IHC. That tumor contained a small number of cells with *ALK*-split signals showing a much wider distance than would be expected for *EML4-ALK* fusion. The RT-PCR study of the kinase domain of *ALK* in this case showed no expression of the *ALK* gene, virtually ruling out any unknown functional translocation involving *ALK* (data not shown), and the significance of this FISH result is unclear. Although some previous studies using FISH assay with commercially available probes appeared to yield results that were more concordant with RT-PCR or IHC [6,11], our data call attention to the inherent difficulty attached to this modality, and emphasize the need for caution when integrating FISH into routine diagnostics for *ALK*-rearranged lung cancer. The development of smaller customized probes may be helpful for more reliable detection of this genetic change.

The *EML4-ALK* fusion gene detected in patient 9 in the present series is a novel variant, in which *EML4* exon 14 was joined with part of exon 20 at a point 36 nucleotides distal to the beginning of exon 20 of *ALK*. This transcript would be read in-frame to generate an intact chimeric protein that maintains the kinase domain of the *ALK* gene, and would be expected to result in overactivation of the signaling pathway downstream to ALK. The literature regarding the nomenclature of the *EML4-ALK* variants is somewhat confusing; seven variants (1, 2, 3a, 3b, 4, 5a, 5b, 6, and 7) were discovered by the same group of investigators, while two different groups have independently identified variants "4" and "5", respectively [2]. Recently, two more variants were added [14], and thus, to our knowledge, the current variant is the 12th. There are no conclusive data to indicate whether different variants are associated with different clinical or histological features.

One of the tumors shown to be positive for ALK alteration by FISH and IHC exhibited focal squamous differentiation. Although most of the previous reports have documented the exclusive adenocarcinoma histology of *ALK*-rearranged tumors, a small number have been reported to show squamous differentiation [5,6,11,16]. The current additional case further reinforces the view that the presence of *EML4-ALK* fusion is not restricted to a pure adenocarcinoma histology. Wong et al. [16] have even identified an *EML4-ALK* fusion gene in a tumor that was interpreted as mucoepidermoid carcinoma. Notably, the combination of solid growth, uniform lower-grade nuclei, clusters of mucin-rich cells, frequent diffuse p63 immunoreactivity, and rare unequivocal squamous differentiation seen in our present series of Ad-SRCCs imparted a superficial resemblance to mucoepidermoid carcinoma. However, a coexisting typical acinar or papillary growth pattern of adenocarcinoma, lack of endobronchial growth, and TTF-1 immunopositivity readily ruled out that possibility. *CRTC1-MAML2* or *CRTC3-MAML2* translocations associated with mucoepidermoid carcinomas [37] were not identified by RT-PCR in any of the present Ad-SRCCs (cases 1–9) (data not shown).

ALK-translocation-positive Ad-SRCCs in this series lacked mutations of either *EGFR* or *KRAS*, confirming the prior observations that *ALK* alteration is mutually exclusive of such genetic events [5,8,9,12,13,16]. What is particularly interesting here is that there was also a total absence of *EGFR* and *KRAS* mutations in the Ad-SRCCs without *ALK* translocations. Considering the high frequency (up to 71% [32]) of *EGFR* or *KRAS* mutations of lung adenocarcinomas in the Japanese population, our findings appear to suggest the unique genetic background of Ad-SRCC of the lung, desplte the admittedly small number of cases studied. It is possible that a certain pathway downstream to the EML4-ALK chimeric protein plays a critical role in creation of the signet-ring cell morphology, and the same pathway may function even in Ad-SRCCs without *ALK* fusion genes. Alternatively, Ad-SRCCs may originate from a certain type of cell that is programmed to differentiate to a signet-ring cell phenotype, and such cells may be somehow more prone to accumulate *ALK* alterations than *EGFR* or *KRAS* mutations. It is unlikely that *EML4-ALK* itself determines the signet-ring cell cytology, because not all of the *ALK*-translocation-positive adenocarcinomas of the lung showed this particular cell type [11]. It is noteworthy that certain clinical features (younger age at onset and minimal tobacco exposure) are shared by both Ad-SRCCs and *ALK*-rearranged tumors [8,12,21,22], possibly suggesting an inherent close relationship between the two. A few previous reports have detected *KRAS* mutations in some Ad-SRCCs [24,26], and this discrepancy may be due to the small number of cases examined, or differences in the criteria used to select the Ad-SRCCs.

In conclusion, this study has confirmed the previously observed association between Ad-SRCC and *ALK*-rearrangement. The characteristic histology, immunoprofile (TTF-1/p63 co-expression), frequent *ALK* translocation, and total lack of *EGFR* or *KRAS* mutations, may suggest that Ad-SRCC forms a coherent subgroup of lung adenocarcinomas.

Conflict of interest

None declared.

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The Impact of Superior Mediastinal Lymph Node Metastases on Prognosis in Non-small Cell Lung Cancer Located in the Right Middle Lobe

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Background: We aimed to assess hilar and mediastinal lymph node involvement and its impact on prognosis in patients with right middle lobe lung cancer.

Methods: The records of 170 patients undergoing surgery for right middle lobe non-small cell lung cancer from 1980 to December 2007 were retrospectively examined. There were 45 patients found to have hilar or mediastinal lymph nodes metastases. This subgroup included 31 N2 patients and 14 N1 patients, and included 23 women and 22 men, whose ages ranged from 32 to 83 years (median = 61 years). The status of mediastinal, hilar, and interlobar lymph nodes was assessed according to the seventh edition of the TNM classification for lung cancer. Patient records were examined for age, gender, preoperative nodal status, surgical procedure, metastatic status of lymph nodes (distribution and numbers), tumor size, and histologic features (cell type and differentiation degree). Survival duration was defined as the interval between surgery and death from the tumor or the most recent follow-up.

Results: For N1 cases (n = 14), the most frequent metastatic site was #12m (lymph nodes adjacent to the middle lobe bronchus), which occurred in 11 cases; there was one case with metastases in #11s (lymph nodes between the upper lobe bronchus and bronchus intermedius), and no case with #11i metastases (lymph nodes between the right middle and lower lobe bronchi). The most frequent metastatic mediastinal zone was the subcarinal zone (25/31), and the superior mediastinal zone also had a high incidence of metastases (22/31). Sixteen cases had metastases to both the superior and subcarinal zones, and six cases had metastasis to superior mediastinal zone without subcarinal zone metastasis. When #11s or #11i was involved, eight of nine or five of five, respectively, were N2 cases. Univariate analyses revealed that tumor diameter, cN, status of lymph node metastases, and operative procedure (pneumonectomy) were significant prognostic factors in N2 cases. Regarding status of lymph node metastases, superior mediastinal zone metastases, both superior and inferior (subcarinal) zone metastases, and #11i were significant prognostic factors. Because #11i metastases and superior mediastinal lymph nodes metastases were highly correlated with each other (p = 0.02), two separate models were used in multivariate analyses. Superior mediastinal metastases (p = 0.03) and #11i metastases (p = 0.015) were revealed to be significant independent prognostic factors, whereas multiple-zone metastases only tended toward significance as an adverse prognostic factor (p = 0.054).

Conclusions: Superior mediastinal lymph node metastases and #11i metastases were significant adverse prognostic factors in patients with middle lobe lung cancer, and they were associated with each other.

Key Words: Middle lobe cancer, Superior mediastinal lymph node metastasis, N2, NSCLC.

(*J Thorac Oncol.* 2011;6: 494–499)

The right middle lobe is the smallest lobe in the lung, and lung cancer originating there is much less common than in the other lobes, occurring in 3.8 to 6.7% of all lung cancers.[1–4] The fact that it is less common may be a reason that there are a few reports on the prognostic factors of middle lobe lung cancer.

Lymph drainage from the middle lobe extends to both superior and inferior mediastinal lymph nodes, and previous reports have demonstrated a high incidence of metastases to both the superior and inferior mediastinal zones.[1–6] Nevertheless, there are few articles on the relationships between status of hilar and mediastinal lymph node metastases and patient prognoses.

In this retrospective study, we aimed to clarify prognostic factors in patients with middle lobe lung cancer who underwent surgery. Furthermore, we wanted to determine the association between the status of lymph node metastases and postoperative prognosis.

PATIENTS AND METHODS

This was a retrospective study. Because individual patients were not identified, our institutional review board waived the requirement for obtaining patient consent and approved this study. Between 1980 and December 2007, 170 patients underwent surgical resection at the Cancer Institute

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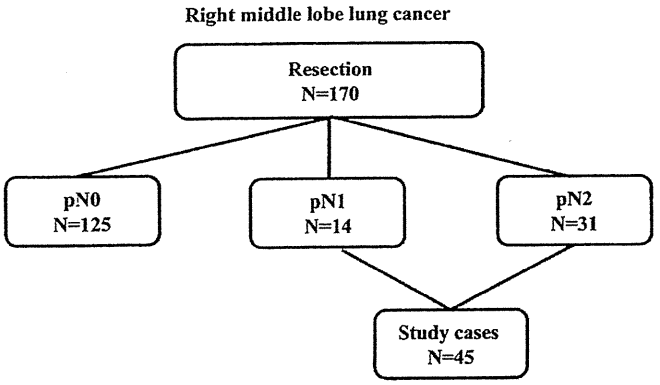

FIGURE 1. Study group subdivisions. Between 1980 and December 2007, 170 patients underwent surgical resections for right middle lobe lung cancer at the Cancer Institute Hospital. There were 14 N1 cases and 31 N2 cases evaluated.

Hospital for primary lung cancer originating in the right middle lobe. Among these patients, 45 were diagnosed with N1 or N2 disease after lung resection and hilar and mediastinal node dissections (Figure 1). The extent of lymph node dissection was not affected by a suspicion of N1 disease. We have routinely performed nearly the same dissection (ND2a).

All the 45 study patients were confirmed for their prognoses. The primary surgical procedure for lymph node dissection, such as hilar and mediastinal nodal dissection, was established in Japan in the late 1970s. In our institute, the extent of lymph node dissection conducted recently is nearly the same as that during the 1980s. Some cases had sampling due to disorders such as cardiac or pulmonary, and these cases were excluded from this study. The resected lymph nodes were separated according to the map[7] in the operating room by the surgeons. Station 10 nodes dissected in middle lobe cancer were adjacent to the inferior parts of the main bronchus, and these nodes were included in the subcarinal zone according to the new TNM. [7] The other station 10 nodes, which were adjacent to the upper parts of the main bronchus, were not routinely dissected, and this area is difficult to dissect without an upper lobectomy.

This subgroup included 31 N2 patients and 14 N1 patients, and included 23 women and 22 men, whose ages ranged from 32 to 83 years (median = 61 years, Table 1). For all patients, preoperative staging was performed using chest computed tomography (CT), abdominal CT or ultrasonography, brain CT or magnetic resonance imaging, and bone scans. Clinical mediastinal and hilar lymph node status was assessed as positive if the chest CT showed that the short axis of a node was more than 1.0 cm. CT scans have been used for evaluating lung cancer staging in our institute since 1980. Of course, CT imaging quality is different when comparing that in the 1980s with that in the 2000s. Nevertheless, this study focused on pathological N status of middle lobe lung cancer, and the quality of pathological examinations was nearly the same during the study period. We excluded those patients who had induction therapy because it seemed to be difficult to evaluate their pathological node status.

TABLE 1. Patient Characteristics

Age (yr)	32–83, median: 61
Gender (male/female)	22/23
c-N	
N0/N1/N2	23/14/8
c-T	
T1/T2/T3/T4	17/24/3/1
p-N	
N1/N2	14/31
Histologic type	
Adenocarcinoma/others	35/10
Well-differentiated/others	10/35
Surgical procedure	
Lobectomy/bilobectomy/pneumonectomy	21/14/10

Bulky N2 (shortest mediastinal lymph node diameter >2 cm) patients have not been candidates for surgery in our institute. Although mediastinoscopy, 18F-fluorodeoxyglucose positron emission tomography, or endobronchial ultrasound with transbronchial needle aspiration was applied to some patients in this series, they were not used for preoperative staging. Follow-up periods ranged from 2 to 302 months (median follow-up for living patients was 86 months).

The status of mediastinal, hilar, or interlobar nodes was assessed according to the seventh edition of the TNM classification for lung cancer.[7] Mediastinal nodes were classified into the following three zones: superior, subcarinal, and inferior. N1 nodes were classified into two zones as hilar or interlobar, and peripheral. The interlobar zone was divided into three subgroups as follows: #12m, lymph nodes adjacent to the middle lobe bronchus; #11s, lymph nodes between the upper lobe bronchus and bronchus intermedius; and #11i, lymph nodes between the right middle and lower lobe bronchi. When a case had mediastinal nodal involvement of two or more zones, it was classified with multiple-zone metastases.

Patient characteristics are summarized in Table 1. Patient records were examined for age, gender, preoperative nodal status, surgical procedure, metastatic status of lymph nodes (distribution and numbers), tumor size, and histologic features (cell type and degree of differentiation).

Statistical Analysis

Survival duration was defined as the interval between surgery and death from the tumor, or the most recent follow-up. Survival rates were calculated using the Kaplan-Meier method. Univariate analyses were performed using the log-rank test, χ^2 test, and logistic regression. Multivariate analyses were performed for variables with p values less than 0.1 by univariate analysis, using the logistic regression test in StatView J 5.0 (SAS Institute Inc., Cary, NC). A p value less than 0.05 was considered significant.

RESULTS

Status of Lymph Node Metastases

In N1 cases (n = 14), the most frequent metastatic site was #12m, occurring in 11 cases, and there was one case with metastases in #11s and 0 cases with #11i metastases (Figure 2).

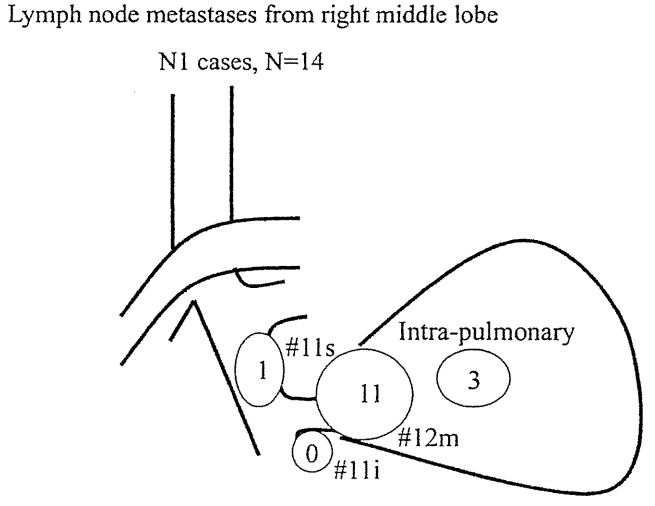

FIGURE 2. Distribution of metastatic nodes in N1 cases.

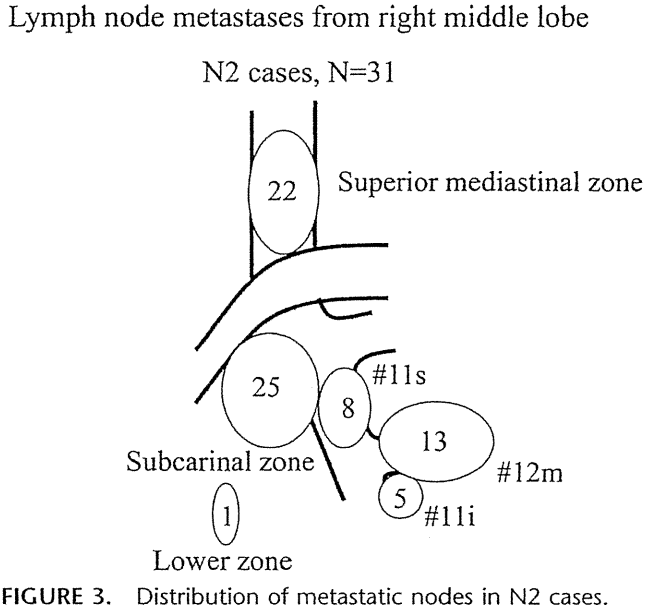

FIGURE 3. Distribution of metastatic nodes in N2 cases.

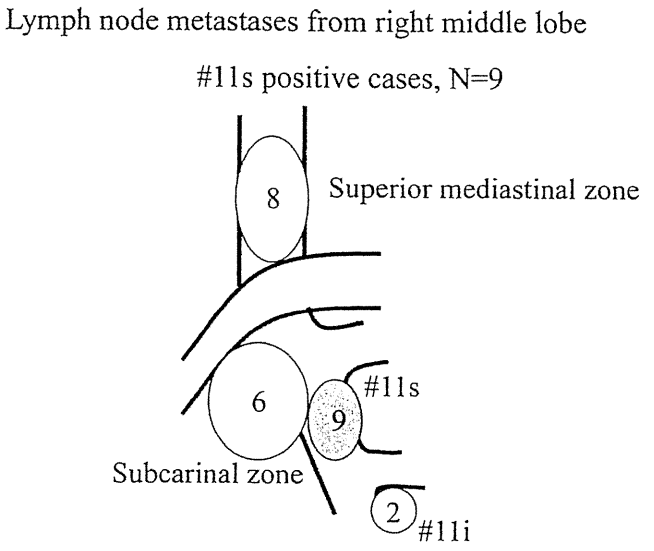

FIGURE 4. Association of #11s metastases with mediastinal zone metastases.

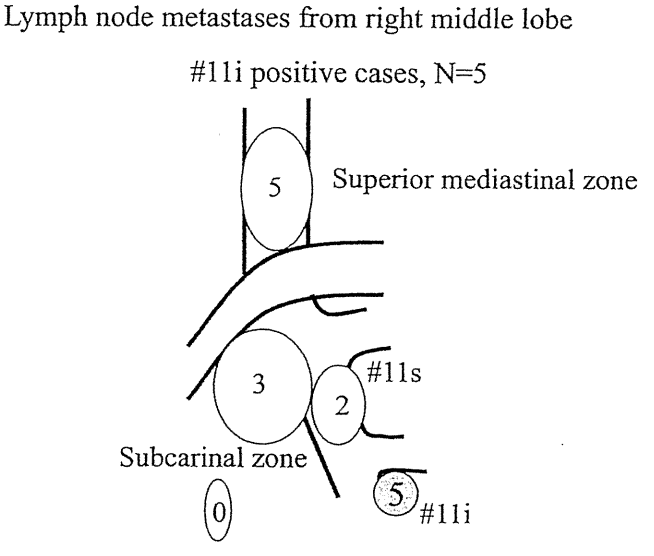

FIGURE 5. Association of #11i metastases with mediastinal zone metastases.

The most frequent metastatic mediastinal zone was the subcarinal zone (25/31 N2 cases). The superior zone also had a high incidence of metastases (22/31 cases). There were 16 cases with metastases in both the superior and subcarinal zones; nine cases were metastasized to the subcarinal zone without the superior mediastinal zone metastasis, and six cases were metastasized to superior the mediastinal zone without the subcarinal zone metastasis (Figure 3). When #11s was involved, eight of nine cases were N2, and when #11i was involved, all five cases were N2 (Figures 4 and 5).

Survival Rates for Patients with Nodal Involvement

The postoperative 5-year survival rate for patients with N1 was 62% and with N2 was 20% ($p = 0.02$). The postoperative 5-year survival rate was 83% for 125 N0 patients. The prognoses for N0 patients with right middle lobe cancers were not different from those of N0 patients with other involved lobes.

Prognostic Factors for N2 in the Right Middle Lobe

Univariate analyses using the variables listed in Table 2 showed that diameter, cN1–2/cN0, status of lymph node metastases, and operative procedure (pneumonectomy) were significant prognostic factors. Nevertheless, there was no difference in prognoses between lobectomy and bilobectomy. Regarding specific prognostic lymph node metastases, superior mediastinal zone metastases, both superior and subcarinal and interlobar #11i metastases were significant prognostic factors. Inferior mediastinal zone metastases, and #12m and

TABLE 2. Prognostic Factors for Patients with N2: Univariate Analysis

Variables	Cases	5-yr Survival (%)	*p*
Gender			
Male/female	14/17	17.8/22.0	0.95
Age			
<70 yr/70 yr or older	22/9	21.7/16.0	0.37
Diameter (14–65 mm, mean: 35 mm)			
<35 mm/35 mm or larger	16/15	.36.5/6.8	0.02
cN			
cN1–2/cN0	15/16	8.0/33.7	0.042
cN0–1/cN2	23/8	23.3/12.5	0.24
Adenocarcinoma/others	26/5	25.2/0	0.1
Well differentiated/others	6/25	16.7/21.5	0.81
Pleural involvement yes/no	15/16	21.8/17.3	0.57
Status of lymph node metastases			
Superior mediastinal zone yes/no	22/9	6.4/50.8	0.005
Inferior mediastinal zone yes/no	25/6	21.4/16.7	0.61
#12m yes/no	13/18	24.7/18.3	0.92
#11s yes/no	8/23	14.3/21.9	0.14
#11i yes/no	5/26	0/23.6	0.02
Both superior and inferior zones			
Multiple zones/single zone	16/15	0/36.4	0.01
Operative procedure			
Pneumonectomy vs. others	7/24	0/25.8	0.009
Lobectomy vs. bilobectomy	16/8	23.6/29.2	0.61
Period			
Before 1995 vs. from and after 1995	15/16	13.3/28.4	0.28

#12m, lymph nodes adjacent to middle lobe bronchus; #11s, lymph nodes between the upper lobe bronchus and bronchus intermedius; #11i, lymph nodes between the right middle and lower lobe bronchi.

TABLE 3. Prognostic Factors for Patients with N2: Multivariate Analysis

Variables	Odds Ratio	95% CI	*p*
Model 1			
Diameter	1.04	0.99–1.08	0.054
cN			
cN1–2/cN0	1.87	0.71–4.95	0.21
Status of lymph node metastases			
Superior mediastinal zone	5.08	1.20–21.8	0.03
Multiple zones	1.42	0.51–3.96	0.50
Operative procedure			
Pneumonectomy	1.13	0.30–4.34	0.88
Model 2			
Diameter	1.03	0.99–1.06	0.17
cN			
cN1–2/cN0	1.38	0.54–3.52	0.51
Status of lymph node metastases			
#11i	4.80	1.34–17.0	0.015
Multiple zones	2.80	0.98–7.96	0.054
Operative procedure			
Pneumonectomy	2.32	0.62–10.0	0.20

#11i, lymph nodes between the right middle and lower lobe bronchi.

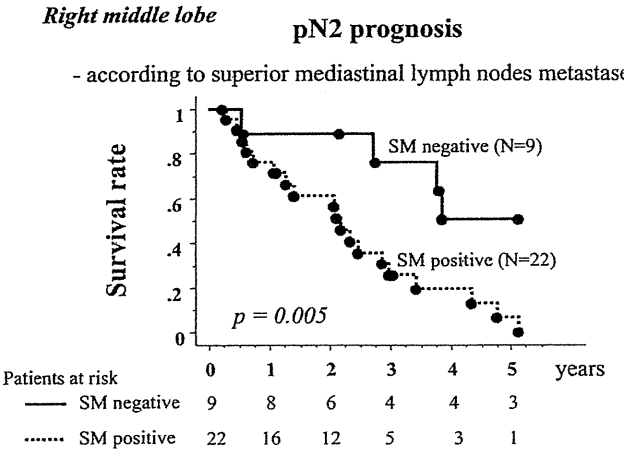

FIGURE 6. Postoperative survival according to superior mediastinal nodal involvement.

#11s metastases were not significant. There was no difference in prognoses between the patients before 1995 and patients from 1996 and after (5-year survivals of 13.3% and 28.4%; $p = 0.28$).

Significant variables by univariate analyses were analyzed by multivariate analyses (Table 3, models 1 and 2). Because #11i metastases and superior mediastinal lymph nodes metastases were highly correlated with each other ($p = 0.02$), two separate models were used for multivariate analyses. In model 1, superior mediastinal metastases were revealed to be a significant independent prognostic factor ($p = 0.03$). In model 2, #11i metastases were revealed to be a significant independent prognostic factor ($p = 0.015$), whereas multiple zone metastases only tended toward significance as an adverse prognostic factor ($p = 0.054$).

Survival Rate According to Prognostic N2 Factors

N2 patients were categorized according to whether they had significant prognostic factors determined from multivariate analyses, including superior mediastinal lymph nodes metastases, #11i metastases, or multiple mediastinal metastatic zones.

The 5-year survival rate was 50.8% in patients without superior mediastinal lymph nodes metastases, whereas it was 6.4% in patients with superior mediastinal lymph node metastases ($p = 0.005$, Figure 6). The 5-year survival rate was 23.6% in patients without #11i lymph node metastases, whereas there were no long-term survivors (dead within 3 years) in patients with #11i lymph node metastases ($p = 0.008$, Figure 7). Furthermore, the 3-year and 5-year survival rates were 58.2% and 36.4% in patients with single-zone mediastinal lymph node metastases, whereas they were 29.6% and 0% in patients with multiple-zone mediastinal lymph node metastases ($p = 0.01$), respectively. Nevertheless, by multivariate analysis, superior mediastinal lymph