

The seventh edition of the TNM classification for NSCLC<sup>5</sup> has been updated, with some modifications from the sixth edition.<sup>6</sup> However, the LN descriptor in the new classification remains the same as in the previous edition, and depends solely on the anatomic extent of LN involvement, despite the changes in the nodal map. In some other solid tumors, such as breast, gastric, and colorectal tumors, the number of metastatic lymph nodes has been included in the TNM staging system.

In our previous report,<sup>7</sup> we demonstrated that resection of  $\geq 10$  LNs influenced survival and that the number of involved LNs (four and more) is a strong independent prognostic factor in NSCLC. This may provide new information for determining the N category in the next TNM classification. However, it remains unknown whether the total number or anatomic location of involved LNs is a superior prognostic factor in NSCLC. Therefore, we retrospectively compared or combined the number of metastatic LNs (nN) category and the classic pathologic LNs (pN) category on survival in patients with completely resected NSCLC in whom  $\geq 10$  LNs had been harvested.

## MATERIALS AND METHODS

### Patient Eligibility

A total of 1,311 consecutive patients who underwent surgical resection for primary lung cancer at our institution from 2000 to 2007 were examined retrospectively. The patients with clinical stages IA to IIIA, including patients with stage cN2 with single-station nodal metastasis, underwent surgery. Cases of induction therapy, incomplete resection, and limited resection were excluded from this study. Patients with tumors classified histologically as small-cell lung cancer or low-grade malignancies were also excluded. In addition, those in whom nine or fewer LNs were harvested were also excluded in the present analysis because our previous study suggested that resection of at least 10 LNs was necessary to maintain the optimal quality of surgery and accurate staging.<sup>7</sup> Finally, a total of 689 patients with NSCLC who underwent complete resection involving dissection of  $\geq 10$  hilar and mediastinal lymph

nodes with curative intent, consisting of lobectomy or more extensive resection, were eligible.

### Data Collection

The patient charts, including the pathologic diagnosis and operative reports, were reviewed. Staging was determined according to the sixth edition of the TNM staging system.<sup>8</sup> The histologic tumor type was determined according to the World Health Organization classification (third edition).<sup>9</sup> LNs were dissected with the adipose connective tissue of the corresponding anatomic regions, as designated by the surgeon intraoperatively. All dissected LNs were examined pathologically and classified on the basis of anatomic location by the numbering system described in the Naruke map.<sup>10</sup> The number of resected and involved LNs from each defined anatomic location was confirmed on the basis of the pathologic report provided by Drs Nomura, Matsubayashi, and Nagao. We performed two different stratifications of LN status assessment: the absence or presence and anatomic extent of nodal metastases (pN categories), and the number of regional LNs with metastases (nN categories). Based on our previous results, four or more involved LNs is the best benchmark of prognostic variables.<sup>7</sup> Therefore, we classified involved LNs into the three nN categories as follows: nN0, no LN metastasis; nN1-3, metastasis in one to three nodes; and nN4+, metastasis in four or more LNs. The pathologists were blinded to the clinical outcome.

We chose overall survival (OS) and disease-free survival (DFS) as end points and investigated the associations between the nN categories and these endpoints compared with standard pN categories. OS was calculated from the date of surgery to the time of death. Observations were censored at final follow-up if the patient was alive. DFS was defined as the time from surgery to locoregional relapse or distant metastasis of lung cancer, and in cases without relapse, any deaths due to causes other than lung cancer were censored. Patients were examined at intervals of 3 months for the first 2 years and at intervals of 6 months for the next 3 years or thereafter on an outpatient basis. The follow-up evaluation involved the following procedures: physical examination, chest radiography, CT scan of the chest and abdomen, and blood examination, including that of pertinent tumor markers. Further evaluations, including brain MRI or CT scan, bone scintigraphy, and integrated PET scan, were performed on the first appearance of any symptoms or signs of recurrence. The median follow-up time was 3.5 years.

### Statistical Analysis

Survival curves were plotted using the Kaplan-Meier method. Differences in survival among the groups were examined using the log-rank test. A two-category comparison was performed using the Student *t* test for quantitative data. Multivariate analysis was performed using the Cox proportional hazards model to examine any possible association between the total number of involved LNs and survival, with adjustment for the effects of other potential prognostic factors, including age, sex, histology, tumor factor, and type of surgery performed. All tests were two-sided, and *P* values of  $< .05$  were considered to indicate statistically significant differences. StatView 5.0 software (SAS Institute Inc) was used for statistical analysis.

### Ethical Considerations

The approval of the institutional review board of Tokyo Medical University was obtained (project approval no. 965). But, as this was a retrospective study, the need to obtain written informed consent from either the patients or their representatives was waived, in accordance with the American Medical Association.

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## RESULTS

### Patient Characteristics

The characteristics of patients were as follows: median age: 64.5 years; sex: 417 men (60.5%) and 272 women (39.5%); histopathologic diagnosis: 497 adenocarcinomas (72.1%), 140 squamous cell carcinomas (20.3%), 42 large cell carcinomas (6.1%), and 10 others (1.5%); pathologic stages: 480 stage I (69.7%), 94 stage II (13.6%), and 115 stage III (14.1%); pN factors: 510 pN0 (74.0%), 93 pN1 (13.5%), and 86 pN2 (12.5%); nN factors: 510 nN0 (74.0%), 101 nN1-3 (14.5%), and 78 nN4- (11.4%). The mean number of resected LNs was 18.1 (right side, 18.5; left side, 17.6). The mean number of involved LNs was 4.5 (range, 1-22) in LN-positive cases (Table 1).

### Survival Analysis

First, we classified the patients into three nN categories: nN0, no LN metastasis; nN1-3, metastasis in one to three nodes; and nN4-, metastasis in four or more LNs. We then assessed the OS and DFS in each pN stage classification and nN category (Fig 1). The survival curves showed clear differences in the OS and DFS of each subgroup of both the pN and nN classifications. There was also a significant difference in OS and DFS for each of the nN categories (the 5-year OS rates for nN0, nN1-3, and nN4- were 79.2%, 64.8%, and 39.2%, respectively,  $P = .0426$  and  $P < .0001$  for nN0 vs nN1-3 and nN1-3 vs nN4-, respectively; the 5-year DFS rates were 83.0%, 71.6%, and 32.9%, respectively,  $P = .0024$  and  $P = .0002$  for nN0 vs nN1-3 and nN1-3 vs nN4-, respectively).

Second, we performed validation of the nN category in terms of OS for each pathologic tumor (pT) category (Fig 2). Although the differences between each pair of nN categories were not always significant, there was a tendency toward the deterioration of OS from the nN0 to the nN4- subgroup. Similar results were found in terms of DFS (data not shown).

Third, we classified the patients into five categories of combinations of the pN and nN status to compare the prognostic significance of the pN and nN status. The five N categories were as follows: pN0-nN0, pN1-nN1-3, pN1-nN4-, pN2-nN1-3, and pN2-nN4-. As shown in Figure 3, patients with pN2-nN1-3 ( $n = 22$ ) had better prognoses than patients with pN1-nN4- ( $n = 13$ ). However, there was no statistically significant difference between these two groups due to the small populations. The survival curve of pN2-nN1-3 patients was similar to that of pN1-nN1-3 patients, which is an operable population, while the survival curves of pN1-nN4- patients were similar, but still superior to that of pN2-nN4- patients.

Because of the strong correlation between the pN and nN categories, we performed multivariate analysis

**Table 1—Patient Characteristics (N = 689)**

Variable/Category	No. (%)
Age, y	
Mean	64.5
Range	26-87
Sex	
Male	417 (60.5)
Female	272 (39.5)
Histology	
Adenocarcinoma	497 (72.1)
Squamous cell	140 (20.3)
Large cell	42 (6.1)
Other	10 (1.5)
Tumor location	
Right	452 (65.6)
Upper/middle/lower	274/31/147
Left	237 (34.4)
Upper/lower	134/103
Surgical procedure	
Lobectomy	637 (92.4)
Bilobectomy	37 (5.4)
Pneumonectomy	15 (2.2)
p Stage	
I	480 (69.7)
II	94 (13.6)
III	115 (14.1)
pT factor	
pT1	344 (50.0)
pT2	283 (41.1)
pT3	27 (3.9)
pT4	34 (5.0)
pN factor	
pN0	510 (74.0)
pN1	93 (13.5)
pN2	86 (12.5)
nN factor	
nN0	510 (74.0)
nN1-3	101 (14.6)
nN4-	78 (11.4)
Total No. of resected LNs	
Mean (range)	18.1 (10-49)
10-19	450 (65.3)
20-29	192 (37.9)
≥ 30	47(6.8)
No. involved LNs in positive cases	
Mean (range)	4.5 (1-22)

LN = lymph node, nN = number of lymph nodes; pN = pathologic lymph node; pT = pathologic tumor.

for each category to confirm each prognostic impact for OS and DFS.<sup>11</sup> On multivariate analysis, the nN category was an independent prognostic factor for OS and DFS (vs nN4-; the hazard ratios of nN0 and nN1-3 for OS were 0.223 and 0.369, respectively,  $P < .0001$  for all categories) as was the case for the pN category (Tables 2, 3). Therefore, both the pN and nN categories were identified as strong prognostic factors for OS and DFS in NSCLC. Moreover, the populations of the pN1-nN1-3 and pN2-nN1-3 categories were small, and the OS of patients within these two groups did not statistically differ. And, there were significant differences between pN1 and pN2

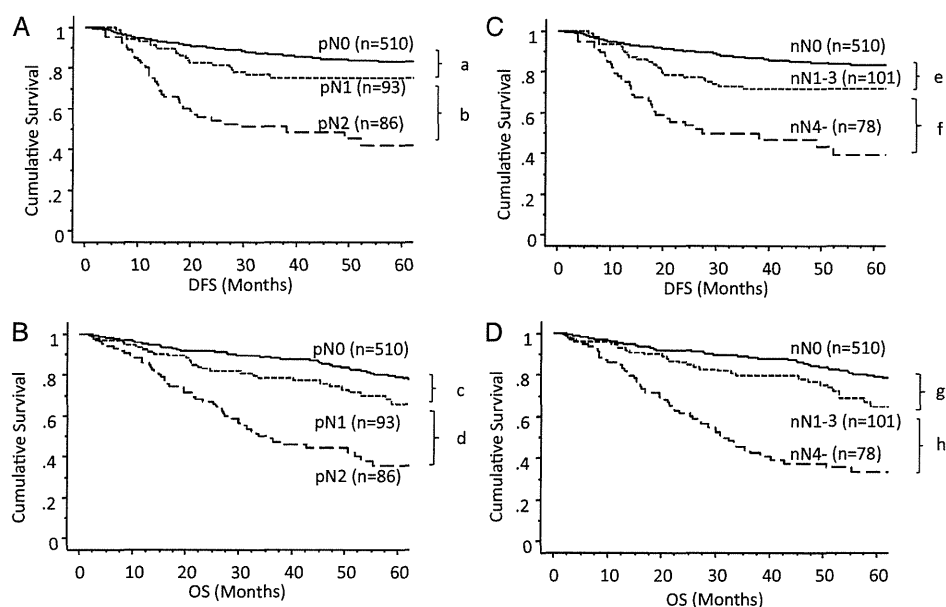


FIGURE 1. DFS and OS according to pN status and nN status. A, DFS curve according to pN status. The 5-year DFS rates for pN0, pN1, and pN2 were 83.0%, 75.3%, and 31.1%, respectively. a, pN0 vs pN1,  $P = .0464$ ; b, pN1 vs pN2,  $P < .0001$ . B, OS curve according to pN status. The 5-year OS rates for pN0, pN1, and pN2 were 79.2%, 65.9%, and 35.4%, respectively. c, pN0 vs pN1,  $P = .0181$ ; d, pN1 vs pN2,  $P < .0001$ . C, DFS curve according to nN status. The 5-year DFS rates for nN0, nN1, and nN2-3 were 83.0%, 71.6%, and 39.2%, respectively. e, nN0 vs nN1,  $P = .0024$ ; f, nN1 vs nN2-3,  $P = .0002$ . D, OS curve according to nN status. The 5-year OS rates for nN0, nN1, and nN2-3 were 79.2%, 64.8%, and 32.9%, respectively. g, nN0 vs nN1,  $P = .0426$ ; h, nN1 vs nN2-3,  $P < .0001$ . DFS = disease-free survival; nN = number of lymph nodes; OS = overall survival; pN = pathologic lymph node.

(Figs 1A, 1B) and between nN1-3 and nN4- (Figs 2A, 2B), which mean by a still subcategory exist. We propose a new classification for testing, based on combined pN and nN categories: namely, the new N0 category becomes pN0-nN0, the new N1 category becomes pN1-nN1-3, the new N2a category becomes pN2-nN1-3 + pN1-nN4-, and the new N2b category becomes pN2-nN4. Figure 4 shows the survival curves of the new classifications, which were proportional and well distributed among the curves.

## DISCUSSION

The TNM stage classification was developed to provide high specificity for patients with similar prognoses and treatment options. Nodal status is a major determinant of stage and survival of patients with NSCLC after surgery. The seventh TNM staging system included notable changes in the T and M descriptors and in the nodal map, while the N descriptor remained the same as in the previous version and depended solely on the anatomic extent of involved LNs. The anatomically based pN classification has some unsatisfactory aspects. Of these, the heterogeneity of pN1 and pN2 with regard to prognosis is the most notable. Therefore, some subclassifications have been proposed.<sup>1-4,12-17</sup> In addition, differences among surgeons in the labeling of LN stations intraoperatively

will occur regardless of the use of a new nodal map. This indicates that it is necessary to refine the currently used pN stage classification and has justified attempts to identify alternative nodal classification methods. In some other solid tumors, such as breast, gastric, and colorectal tumors, the number of metastatic lymph nodes has been included in the TNM staging system. The number of metastatic LNs, when classified into several categories, has been shown to be a prognostic factor for resected NSCLC.<sup>11,15,18</sup> Wei and colleagues<sup>11</sup> evaluated this issue and suggested that the nN category is a better prognostic determinant than the location-based pN stage classification. However, to date, it has remained unknown whether the nN category or the pN stage classification is a better prognostic factor in lung cancer.

It is important to consider how many or to what extent LNs should be harvested for the accurate assessment of nodal status and to maintain the optimal quality of surgery in NSCLC before evaluating the effectiveness of prognostic determinants among the pN and nN categories. The number of resected LNs in early NSCLC has been proven to be a prognostic factor which has influenced survival, similar to that in colorectal, breast, and bladder cancer.<sup>19-24</sup> Some reports have suggested that the optimal number of removed LNs is 11 to 16 in order to accurately assess stage I lung cancer.<sup>24,25</sup> In another study, the removal

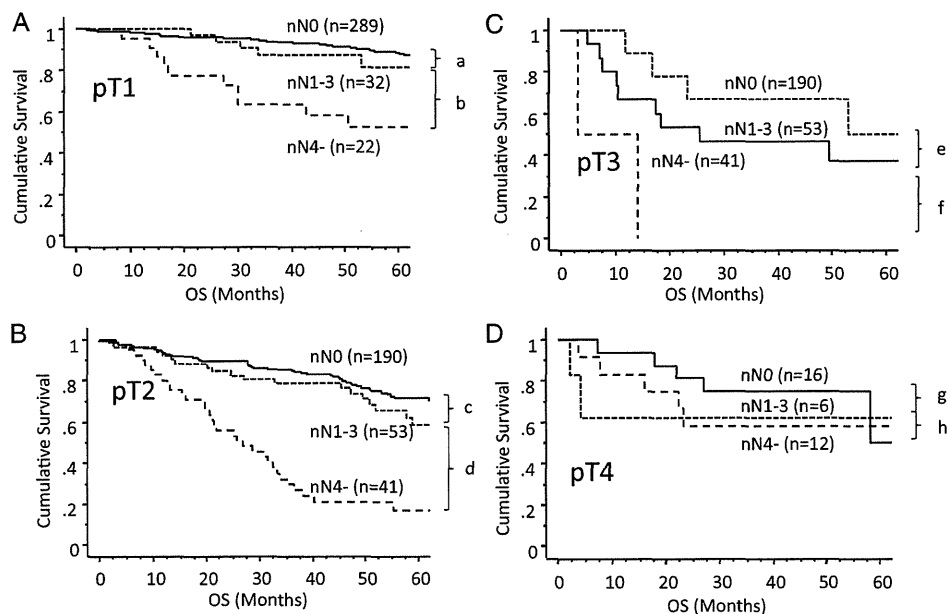


FIGURE 2. OS curves according to nN status across each pT category. A, OS curve according to nN status in pT1 patients. The 5-year OS rates for nN0, nN1, and nN2-3 were 88.5%, 81.4%, and 52.1%, respectively. a, nN0 vs nN1-3,  $P = .6757$ ; b, nN1-3 vs nN4-,  $P = .0024$ . B, OS curve according to nN status in pT2 patients. The 5-year OS rates for nN0, nN1-3, and nN4- were 71.2%, 58.7%, and 16.7%, respectively. c, nN0 vs nN1-3  $P = .6083$ ; d, nN1-3 vs nN4-  $P < .0001$ . C, OS curve according to nN status in pT3 patients. The 5-year OS rates for nN0, nN1-3, and nN4- were 37.3%, 50.0%, and 0%, respectively. e, nN0 vs nN1-3,  $P = .2537$ ; f, nN1-3 vs nN4-,  $P = .0046$ . D, OS curve according to nN status in pT4 patients. The 5-year OS rates for nN0, nN1-3, and nN4- were 50.0%, 62.5%, and 58.3%, respectively. g, nN0 vs nN1-3  $P = .4305$ ; h, nN1-3 vs nN4-,  $P = .8623$ . pT = pathologic tumor. See Figure 1 legend for expansion of other abbreviations.

of 11 LNs was set as a threshold for inclusion.<sup>18</sup> The Staging Manual in Thoracic Oncology of the International Association for the Study of Lung Cancer (IASLC) recommends that at least six LNs/stations be removed or sampled and histologically confirmed to be free of disease in order to define pN0 status.<sup>5</sup> We previously demonstrated that the resection of 10 or more LNs influenced survival while maintaining the quality of surgery.<sup>7</sup> Therefore, in the present analysis, we excluded those for whom  $< 10$  LNs were harvested. In the present series, 617 of 689 cases (89.6%) met this criterion. In the TNM classification for some other tumors, the number of positive LNs has been included in the definition of pN categories.<sup>26</sup> The number of metastatic LNs, when classified into several categories, has been shown to be a prognostic factor for resected NSCLC.<sup>11,15,18</sup> There was a significant difference in OS and DFS among each nN category as well as the pN categories. The OS and DFS survival curves of each nN category are well distributed and proportional (Fig 1). Moreover, as Figure 2 shows, a clear tendency toward the deterioration of OS from nN0 to nN4- in the same pT category was observed when we attempted to validate the results for each pT category. The curves were evenly distributed over pT1, pT2, and pT3. However, the curves were closer

in the higher pT stage of pT4, perhaps due to the small population size. Another reason may be that the prognosis of the higher pT category was already poor, regardless of the presence of metastatic LNs. On multivariate analysis, not only the pN status, but also the nN status, was demonstrated to be a major independent prognostic factor for both OS and DFS in the current series, which is consistent with a previous report.<sup>11</sup> These results showed that both pN and nN categories have a powerful discriminative ability concerning the prognosis of NSCLC.

In general, patients with NSCLC with pN1 or pN2 disease are known to exhibit prognostic heterogeneity.<sup>1-4,12-17</sup> The OS and DFS curves of pN1 and pN2 were widely distributed in the current series, indicating that there are some subclassifications required to distinguish the two curves. To evaluate these subgroups and demonstrate which is the most accurate prognostic factor, the anatomic location of involved LNs, or the total number of involved LNs, we classified the patients into five categories combining the pN and nN status as follows: pN0-nN0, pN1-nN1-3, pN1-nN4-, pN2-nN1-3, and pN2-nN4-. Patients with pN2-nN1-3 ( $n = 22$ ) had better prognoses than patients with pN1-nN4- ( $n = 13$ ). However, there was no statistically significant difference between the

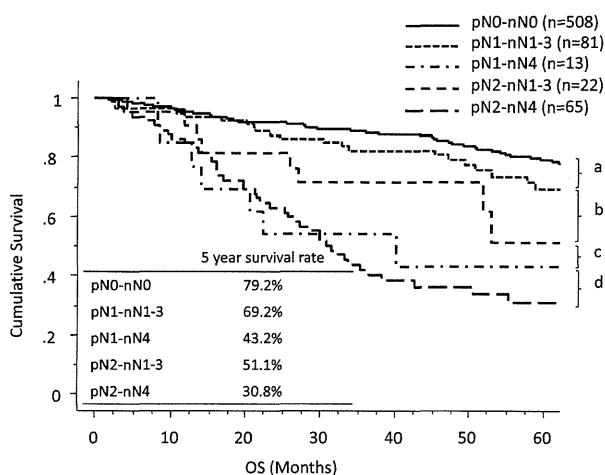


FIGURE 3. OS curves according to combinations between nN status and pN status. Patients with pN2-nN1-3 (n = 22) had better prognoses than patients with pN1-nN4- (n = 13). However, there was no statistically significant difference between the two groups due to the small populations. The survival curve of pN2-nN1-3 was similar to that of pN1-nN1-3, while the survival curves of pN1-nN4- were similar to that of pN2-nN4, a population with worse prognoses. a, pN0-nN0 vs pN1-nN1-3,  $P = .2908$ ; b, pN1-nN1-3 vs pN2-nN1-3,  $P = .1102$ ; c, pN2-nN1-3 vs pN1-nN4,  $P = .1292$ ; d, pN1-nN4- vs pN2-nN4,  $P = .7810$ . See Figure 1 legend for expansion of abbreviations.

two groups due to the small numbers of patients. This result indicates that the nN category might be used to further subdivide the pN category into two prognostically distinct subgroups. Finally, we propose combining patients with pN2-nN1-3 with those who have a better prognosis into the pN2 category and patients in the pN1-nN4- category with those who have a worse prognosis into a single pN category. Therefore,

we reclassified the patients in the current series into four categories as shown in Figure 4. Each OS survival curve of the new classification appears to be proportional with a significant tendency to differ between the new N1 and new N2a and between the new N2a and new N2b categories ( $P = .0028$  and  $P = .0726$ , respectively).

When we subdivided the pN1 and pN2 categories into two subgroups according to the nN category, there was no statistically significant difference between the two groups, but patients with pN2-nN1-3 had better prognoses than patients with pN1-nN4-. This result indicates a possible limitation of the present pN classification for nodal status. The overall disease burden, rather than the anatomic location of LN involvement, has the most relevance in prognosis.<sup>11,27</sup> However, the present pN classification is a major independent prognostic factor in operative NSCLC, as was the nN classification on multivariate analysis in the present series. Therefore, we propose a new nodal classification combination of the pN (anatomic location) and nN (total number) status of LN involvement, which may reflect the survival of operable NSCLC cases more accurately than any single category.

There are some limitations in this study, despite the benefits of the addition of the nN category for predicting survival. First, this was a retrospective and single-institution analysis. Second, it is difficult to accurately estimate the number of LN sites involved both preoperatively and in inoperable patients by CT scan or any other diagnostic imaging methods. The scope of this study involved only the definition of prognosis based on the p stage and not on the c stage, which

Table 2—Multivariate Analysis of OS and DFS Including pN Classification

Variable/Category	OS			DFS		
	HR	95% CI	P Value	HR	95% CI	P Value
Age, y						
< 70	...	...	...	...	...	...
≥ 70	1.018	0.759-1.366	.9053	0.928	0.650-1.325	.6806
Sex						
Men	...	...	...	...	...	...
Women	0.768	0.548-1.076	.1245	1.025	0.709-1.480	.8965
Histopathology						
Nonadenocarcinoma	...	...	...	...	...	...
Adenocarcinoma	0.560	0.409-0.768	.0003 <sup>a</sup>	1.063	0.712-1.588	.7653
pT factor						
T4	...	...	< .0001 <sup>a</sup>	...	...	.0002 <sup>a</sup>
T1	0.378	0.205-0.696	.0018 <sup>a</sup>	0.460	0.227-0.935	.0319 <sup>a</sup>
T2	0.863	0.482-1.545	.619	1.074	0.547-2.103	.8366
T3	1.192	0.564-2.522	.212	1.180	0.450-3.093	.7362
pN factor						
pN2	...	...	< .0001 <sup>a</sup>	...	...	< .0001 <sup>a</sup>
pN0	0.274	0.194-0.386	< .0001 <sup>a</sup>	0.257	0.172-0.33	< .0001 <sup>a</sup>
pN1	0.297	0.188-0.468	< .0001 <sup>a</sup>	0.351	0.206-0.599	.0001 <sup>a</sup>

DFS = disease-free survival; HR = hazard ratio; OS = overall survival. See Table 1 for expansion of other abbreviations.

<sup>a</sup>Statistically significant.

**Table 3—Multivariate Analysis of OS and DFS Including nN Classification**

Variable/Category	OS			DFS		
	HR	95% CI	P Value	HR	95% CI	P Value
Age, y						
< 70	...	...	...	...	...	...
≥ 70	1.023	0.764-1.372	1.023	0.919	0.644-1.311	0.6404
Sex						
Men	...	...	...	...	...	...
Women	0.776	0.556-1.082	0.1344	1.016	0.705-1.463	0.9332
Histopathology						
Nonadenocarcinoma	...	...	...	...	...	...
Adenocarcinoma	0.583	0.425-0.799	0.0008 <sup>a</sup>	1.157	0.775-1.728	0.4760
pT factor						
T4	...	...	< .0001 <sup>a</sup>	...	...	< .0001 <sup>a</sup>
T1	0.473	0.256-0.873	0.0167 <sup>a</sup>	0.551	0.268-1.131	0.1040
T2	1.120	0.624-2.010	0.7036	1.319	0.665-2.618	0.4284
T3	2.114	0.977-4.573	0.5730	1.654	0.616-4.447	0.3182
nN factor						
nN4-	...	...	< .0001 <sup>a</sup>	...	...	< .0001 <sup>a</sup>
nN0	0.200	0.141-0.284	< .0001 <sup>a</sup>	0.223	0.146-0.339	< .0001 <sup>a</sup>
nN1-3	0.197	0.123-0.315	< .0001 <sup>a</sup>	0.369	0.219-0.623	0.0002 <sup>a</sup>

See Table 1 and 2 legends for expansion of abbreviations.

<sup>a</sup>Statistically significant.

is a limitation of this investigation. Technical improvements in preoperative evaluation to accurately identify all metastatic LN sites are necessary. Although there are various clinical markers to evaluate potential malignant lesions, there is as yet no reliable method or evidence suggesting that PET scans or tumor markers can definitively indicate malignancy. Therefore, this is the reason why we decided to concentrate on the p stage as a step toward establishing preoperative clinical evaluation. Third, the definition of the optimal category in terms of the number of metastatic lymph nodes needs to be further explored; because

the definitions, and, therefore, the data, differ according to the institution, it is difficult to determine the optimal category definition. Further multiinstitution studies using identical protocols are needed.

### CONCLUSION

The current results demonstrate that combined anatomically based pN and numerically based nN stage classification as proposed in this study is a better prognostic determinant in pN1 and pN2 prognostically heterogeneous patients with NSCLC. Further large-scale cohort studies, including global prospective validation analyses and multiinstitution studies, are warranted to demonstrate the validity of this proposal for the next TNM classification.

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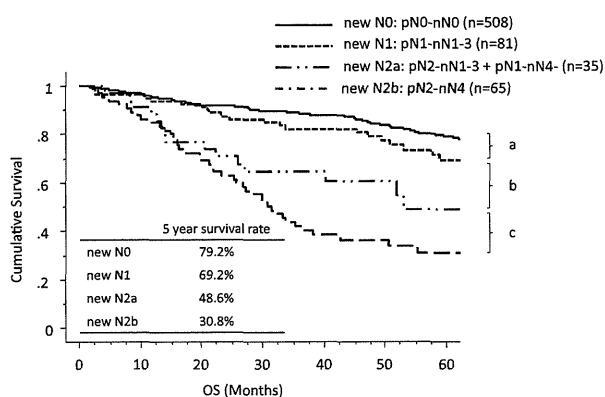


FIGURE 4. OS curves according to combinations of nN status and pN status. We propose a new classification based on combined pN and nN categories: namely, N0 becomes pN0-nN0, N1 becomes pN1-nN1-3, N2a becomes pN2-nN1-3 + pN1-nN4- and N2b becomes pN2-nN4. Each survival curve was proportional and well distributed. a, New N0 vs new N1a,  $P = .2908$ ; b, new N1a vs new N2a,  $P = .0028$ ; c, new N2a vs new N2b,  $P = .0726$ . See Figure 1 legend for expansion of abbreviations.

*Dr Kakahana:* contributed to data collection and analysis and read and approved the final manuscript.

*Dr Usuda:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Kajiwara:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Ohira:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Ikeda:* contributed to preparing the manuscript and read and approved the final manuscript.

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## Prognostic Factors and the Significance of Treatment After Recurrence in Completely Resected Stage I Non-small Cell Lung Cancer

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**Objective:** The objective of this study was to identify the clinicopathologic factors influencing postrecurrence survival (PRS) in and the effect of postrecurrence therapy (PRT) on patients with completely resected stage I non-small cell lung cancer (NSCLC).

**Methods:** We reviewed the data of 919 patients in whom complete resection of stage I NSCLC had been performed.

**Results:** Of the 919 patients, 170 (18.5%) had recurrent disease. Initial PRT was performed in 118 patients (69.1%) (surgery in eight, chemotherapy in 79, radiotherapy in 10, and chemoradiotherapy in 21). On multivariate analyses, PRT (hazard ratio [HR], 0.542; 95% CI, 0.344-0.853;  $P = .008$ ), female sex (HR, 0.487; 95% CI, 0.297-0.801;  $P = .005$ ), and differentiation (HR, 1.810; 95% CI, 1.194-2.743;  $P = .005$ ) demonstrated a statistically significant association with favorable PRS. Bone metastasis (HR, 3.288; 95% CI, 1.783-6.062;  $P < .001$ ), liver metastasis (HR, 4.518; 95% CI, 1.793-11.379;  $P = .001$ ), chemotherapy (HR, 0.478; 95% CI, 0.236-0.975;  $P = .040$ ), epidermal growth factor receptor-tyrosine kinase inhibitors treatment (EGFR-TKIs) (HR, 0.460; 95% CI, 0.245-0.862;  $P = .015$ ), and nonadenocarcinoma (HR, 2.136; 95% CI, 1.273-3.585;  $P = .004$ ) were independently and significantly associated with PRS in the 118 patients who underwent any PRT. Subgroup analysis with a combination of these five PRS factors in the patients who underwent any PRT revealed median PRS times of 42.4 months for 20 patients lacking all five risk factors and 18.8 months for 98 patients with at least one of these risk factors ( $P = .001$ ).

**Conclusions:** PRT, sex, and differentiation were independently associated with PRS. In the patients who underwent any PRT, PRS was related to EGFR-TKIs, chemotherapy, histology, and initial recurrence sites. One challenge for the future will be to create systematic treatment strategies for recurrent NSCLC according to the risk factor status of individual patients.

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**Abbreviations:** EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor; HR = hazard ratio; NSCLC = non-small cell lung cancer; PRS = postrecurrence survival; PRT = postrecurrence therapy; PS = performance status; RFP = recurrence-free proportion

Surgical resection with a curative intent is considered the standard of care for early stage non-small cell lung cancer (NSCLC), but >20% of patients had recurrence, even in pathologic stage I cases.<sup>1,6</sup> Recurrence after complete resection for stages I to III of NSCLC ranges from 30% to 75%, and has been reported to depend on pathologic staging and follow-up period.<sup>1,6-8</sup> The majority of recurrences occur within the first 2 years,<sup>1,6</sup> although there are several studies showing

late recurrences  $\geq 5$  years after resection.<sup>9-11</sup> Long-term, continuous follow-up is required to establish accurate recurrence rates and patterns.

Although several studies focusing on postrecurrence survival (PRS) of patients in stage I or stage I-III NSCLC have been reported,<sup>2-4,8,12-14</sup> no standard treatment strategy for recurrent disease based on prospective studies has been established. However, a standard treatment strategy is necessary because much longer



follow-up periods and robust protocols are required to evaluate PRS objectively. It is difficult to generalize about multifactorial patient backgrounds, which depend on disease, treatment, and performance status (PS) at recurrence. The prognostic factors predicting PRS or the appropriate treatment are still controversial.

Encouraging new treatments (including epidermal growth factor receptor-tyrosine kinase inhibitors [EGFR-TKIs], anaplastic lymphoma kinase inhibitors, pemetrexed, and bevacizumab) have afforded benefits to certain patients with advanced or recurrent NSCLC.<sup>15-21</sup> Advances in postrecurrence therapy (PRT) may provide improvement in overall survival among the patients who undergo surgery. The objective of the present study was to identify the clinicopathologic factors influencing PRS and their effect of PRT on stage I NSCLC.

## MATERIALS AND METHODS

From January 1990 through December 2007, 1,214 patients underwent complete resection for pathologic stage I NSCLC at our hospital. Complete resection was defined as demonstrating cancer-free surgical margins, both grossly and histologically. All patients underwent radical, anatomic, lobar resection and systematic, mediastinal lymph node dissection. The following exclusion criteria were applied: preoperative chemotherapy, radiation therapy, or both ( $n = 38$ ); low-grade malignant tumors, including carcinoids, mucocpidermoid carcinomas, or adenoid cystic carcinomas ( $n = 20$ ); and death within 30 days of operation ( $n = 9$ ). Of the remaining 1,147 patients, complete follow-up was available for 919 patients, who composed the subjects of this study.

Preoperative evaluation included physical examination, chest radiography, CT scan of the chest and abdomen, bone scintigraphy, blood examination, and, since the early 2000s, PET scan (recently integrated PET-CT scan). Histologic subtypes of lung cancer were determined according to the World Health Organization classification,<sup>22</sup> and disease stage was determined in accordance with the *TNM Classification for Lung and Pleural Tumours*, 7th ed.<sup>23</sup>

The follow-up schedule consisted of a clinic visit every 3 months in the first year after resection, every 6 months from the second to the fifth year, and annually thereafter on an outpatient basis, and aimed at continuing follow-up for 10 years after resection. Follow-up procedures included physical examination, chest radiography, and blood examination (including serum tumor markers). CT scans of the chest and abdomen was performed every 6 months in the first 2 years, and annually from the third to the fifth year. Whenever

any symptoms or signs of recurrence were detected, MRI of the brain and bone scintigraphy were performed.

Recurrences were diagnosed by physical examination and diagnostic imaging. Histologic or cytologic confirmation of the recurrence was made when clinically feasible. Local recurrence was defined as disease recurrence at the surgical margin, ipsilateral hemithorax, or mediastinum. Radiographic lymph node recurrence was defined as enlarged lymph nodes measuring  $> 1$  cm on the short axis by CT scan and/or hypermetabolic lymph nodes on PET-CT scans. Pathologic confirmation of recurrence was made by endobronchial ultrasound-guided transbronchial needle aspiration of enlarged lymph nodes during follow-up. Distant metastasis was defined as disease recurrence in the contralateral lung or outside the hemithorax and mediastinum. A second primary tumor was recorded when a patient presented with a new histologic type, and with clinical features consistent with a new primary tumor. Data collected from our department database of patients, telephone interviews, and correspondence from outside sources during the follow-up periods were included.

Clinical characteristics were retrieved from available clinical records. The following clinicopathologic factors were assessed in the PRS analysis: age, sex, smoking status, primary tumor status (T1 vs T2), tumor size (0-30 mm vs  $> 30$  mm), tumor differentiation (well/moderate vs poor), pathologic vascular invasion, pleural invasion, histology (adenocarcinoma vs others), and extent of resection (single lobe lobectomy vs more extensive resection, namely bilobectomy/pneumonectomy).

Length of the recurrence-free period was calculated in months from date of resection to date of initial recurrence or last follow-up showing no recurrence. To calculate the recurrence-free proportion (RFP), patients who died without recognized recurrence or who were known to have no recurrence at the date of last contact were censored. Length of PRS was measured from date of initial recurrence to date of death from any cause or date on which the patient was last known to be alive. PRS and RFP curves were plotted using the Kaplan-Meier method, and differences in variables were determined using the log-rank test or the Breslow tests. Categorical comparison was performed using the  $\chi^2$  test for discrete data and Student  $t$  test for continuous data. Multivariate analyses were performed using the Cox proportional hazards regression model. A backward stepwise selection procedure was implemented. All tests were two-sided, and  $P$  values  $< 0.05$  were considered to indicate a statistically significant difference. Statview 5.0 software (SAS Institute Inc) was used for statistical analyses.

Data collection and analyses were approved, and the need to obtain written informed consent from each patient was waived, by the institutional review board at Tokyo Medical University (No. 2133).

## RESULTS

Median follow-up time for survivors was 62.0 months (range: 1.4-247.6 months). The RFP was 82.2% at 5 years after operation. Of the 919 patients, 170 (18.5%) had recurrent disease, with a median age of 66 years at the time of initial recurrence. Median PRS time for these patients was 17.6 months (range: 0.4-103.0 months). The 1- and 2-year PRS proportions were 73.5% and 51.4%, respectively (Fig 1).

Table 1 shows 5-year RFPs and univariate/multivariate analyses of recurrence according to clinicopathologic characteristics of patients with stage I NSCLC. Univariate analysis identified five significant risk factors:

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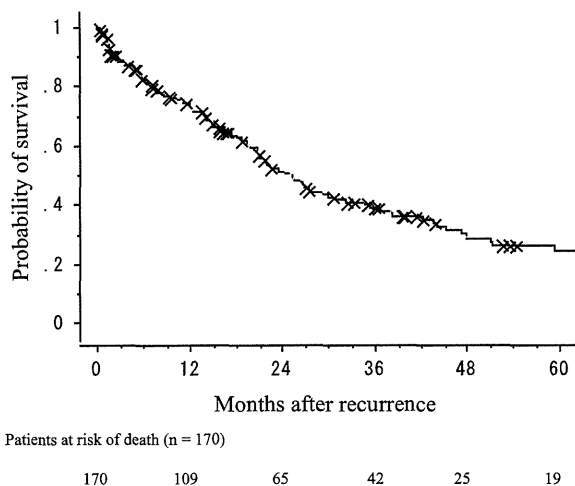


FIGURE 1. Postrecurrence survival curve of patients with non-small cell lung cancer recurrence.

male sex, pathologic vascular invasion, pleural invasion, poorly differentiated carcinoma, and nonadenocarcinoma. Multivariate analysis demonstrated that pathologic vascular invasion (hazard ratio [HR], 2.306;

95% CI, 1.621-3.280;  $P < .001$ ), pleural invasion (HR, 1.489; 95% CI, 1.048-2.115;  $P = .026$ ), and poorly differentiated carcinoma (HR, 1.842; 95% CI, 1.328-2.555;  $P < .001$ ) were statistically significant predictors of recurrence.

Initial recurrence sites and PRT are shown in Table 2. Type of recurrence included only local recurrence in 43 patients (25.3%), distant in 113 (66.5%), and both in 14 (8.2%). Most commonly involved organs were the lung (the site of recurrence in 66 patients: ipsilateral in 23, contralateral/bilateral in 43), followed by regional lymph nodes in 37, brain in 30, bone in 21, and liver in 16. Initial PRT was performed in 118 patients (69.4%), and included surgery for 8, chemotherapy for 79, radiotherapy for 10, and chemoradiotherapy for 21. Surgical resections ( $n = 8$ ) were performed in three patients with solitary pulmonary metastasis, three with solitary brain metastasis, one with adrenal gland metastasis, and one with chest wall and axillary lymph node involvement. Forty-one patients (24.1%) had no treatment for recurrent disease. Of the 118 patients who underwent any PRT, 66 (55.9%) underwent second-line or subsequent therapy, including

Table 1—Patient Characteristics and Univariate and Multivariate Analyses of Recurrence

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	5-y RFP, %	P Value	HR	95% CI	P Value
Age, <sup>a</sup> y						
< 65	439	84.1	...	...	...	...
≥ 65	480	80.4	.129	...	...	...
Sex						
Male	542	78.0	...	...	...	...
Female	377	87.8	<.001	...	...	...
Smoking status						
Never smoker	347	85.2	.134	...	...	...
Ever smoker	572	80.2	...	...	...	...
T category						
T1	512	84.7	...	...	...	...
T2	407	78.9	.100	...	...	...
Tumor size						
0-30 mm	663	84.0	...	...	...	...
> 30 mm	256	81.5	.112	...	...	...
Pathologic vascular invasion						
Absent	481	91.0	...	1	...	...
Present	421	72.1	<.001	2.306	1.621-3.280	<.001
Pleural invasion						
Absent	719	84.9	...	1	...	...
Present	191	71.8	<.001	1.489	1.048-2.115	.026
Histology						
Adenocarcinoma	706	83.8	...	...	...	...
Nonadenocarcinoma	213	76.3	.039	...	...	...
Differentiation						
Well or moderate	656	86.7	...	1	...	...
Poor	216	67.7	<.001	1.842	1.328-2.555	<.001
Type of surgery						
Single lobectomy	873	81.9	...	...	...	...
Bilobectomy or pneumonectomy	46	87.2	.942	...	...	...

HR = hazard ratio; RFP = recurrence-free proportion.

<sup>a</sup>Median age = 65 y.

**Table 2—Initial Recurrence Site and Postrecurrence Therapy**

Data of Recurrence Sites and Postrecurrence Therapies	Patients, No.
Overall	170
Type of recurrence	
Distant	113
Local	43
Both	14
Initial recurrence site	
Ipsilateral lung	23
Contralateral/bilateral lung	43
Regional lymph nodes	37
Malignant effusion/dissemination	13
Stump	9
Brain	30
Bone	21
Liver	16
Adrenal gland	10
Others	14
Postrecurrence therapy	
Initial therapy	
Surgery	8 (lung, 3; brain, 3; adrenal gland, 1; lymph nodes, 1)
Surgery alone	6
Surgery + chemotherapy	3
Chemotherapy	79
Radiation therapy	10
Chemoradiotherapy	21
None	41
Unknown	11
Second-line or the subsequent therapy	66
Chemotherapy	58
EGFR-TKIs	27 (gefitinib, 22; erlotinib, 3; both, 2)
EGFR mutation status/histology	Positive 12 (Ad, 11; Sq, 1) Wild 4 (Ad, 3; LCC, 1) Unknown 11 (Ad, 10; LCC, 1)
Others	7

Ad = adenocarcinoma; EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor; LCC = large cell carcinoma; Sq = squamous cell carcinoma.

chemotherapy for 58, and EGFR-TKIs for 27 (gefitinib, 22 patients; erlotinib, three patients; and both, two patients). Among the latter 27 patients, *EGFR* mutations were detected in 12; four had wild-type *EGFR*.

Table 3 shows univariate/multivariate analyses of PRS. Univariate analysis identified six significant risk factors for PRS: male sex, smoking, poorly differentiated carcinoma, nonadenocarcinoma, no PRT, and shorter recurrence-free interval ( $\leq 24$  months; median recurrence-free period was 24 months). Multivariate analysis demonstrated that PRT (HR, 0.542; 95% CI, 0.344-0.853;  $P = .008$ ), female sex (HR, 0.487; 95% CI, 0.297-0.801;  $P = .005$ ), and differentiation (HR, 1.810; 95% CI 1.194-2.743;  $P = .005$ ) had a statistically significant association with favorable PRS.

The results of multivariate analysis of PRS determined that PRT had a strong impact on PRS. There-

fore, we further examined PRS in the 118 patients who underwent any PRT (Table 4). Univariate analysis identified nine significant risk factors for PRS: male sex, smoking, poorly differentiated carcinoma, bone metastasis, liver metastasis, no chemotherapy or EGFR-TKI, no second-line therapy, and multiple organ metastases. Multivariate analysis demonstrated that bone metastasis (HR, 3.288; 95% CI, 1.783-6.062;  $P < .001$ ), liver metastasis (HR, 4.518; 95% CI, 1.793-11.379;  $P = .001$ ), chemotherapy (HR, 0.478; 95% CI, 0.236-0.975;  $P = .040$ ), EGFR-TKI therapy (HR, 0.460; 95% CI, 0.245-0.862;  $P = .015$ ), and nonadenocarcinoma (HR, 2.136; 95% CI, 1.273-3.585;  $P = .004$ ) had a statistically significant association with PRS.

Subgroup analysis with a combination of these five PRS factors (no EGFR-TKI and chemotherapy, presence of liver or bone metastasis, nonadenocarcinoma) in patients with recurrence who underwent any PRT revealed median PRS times of 42.4 months for 20 patients lacking all five unfavorable factors and 18.8 months for 98 patients with one of these risk factors, respectively (Fig 2). The difference in PRS was statistically significant between the two groups ( $P = .001$ ).

## DISCUSSION

We set out to identify clinicopathologic factors influencing PRS of patients with stage I NSCLC. Although curative surgical resection is the most effective therapy for stage I NSCLC, a considerable number of patients will develop recurrence. In the current study, overall incidence of recurrence was 18.5%, and median PRS time was 17.6 months. Initial location of recurrence was at a distant site in 74.7%, and the proportions of recurrences within 2 or 3 years after surgery were 48.2% and 66.5%, respectively (unpublished data). Previous studies have reported that the incidence of recurrence in patients with stage I NSCLC was 14% to 36%, with the 1-year survival rate ranging from 30% to 68% (Table 5).<sup>1-6,8,24</sup>

We examined risk factors for recurrence in stage I NSCLC, and identified three: pathologic vascular invasion, pleural invasion, and poorly differentiated carcinoma. These standard pathologic factors have also been reported to be good predictors of overall survival for patients with stage I NSCLC.<sup>25-36</sup> In our study, univariate analysis for PRS identified six significant risk factors (male sex, smoking, poorly differentiated carcinoma, nonadenocarcinoma, no PRT, and shorter recurrence-free interval [ $\leq 24$  months]), while multivariate analysis revealed that sex, PRT, and differentiation were independent prognostic factors. Only differentiation was a significant predictor of recurrence and poor PRS, and pathologic vascular invasion and pleural invasion had no significant impact on PRS. PRS may be associated

**Table 3—PRS Analyses**

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	Median PRS, mo	P Value	HR	95% CI	P Value
Age at recurrence, <sup>a</sup> y						
< 66	76	18.9	...	...	...	...
≥ 66	94	15.8	.242	...	...	...
Sex						
Male	118	15.5	...	1	...	...
Female	52	25.6	<.001	0.487	0.297-0.801	.005
Smoking status						
Never smoker	59	25.0	...	...	...	...
Ever smoker	111	14.1	.006	...	...	...
T category						
T1	87	15.8	...	...	...	...
T2	83	19.6	.476	...	...	...
Tumor size						
0-30 mm	132	16.9	...	...	...	...
> 30 mm	38	20.9	.632	...	...	...
Pathologic vascular invasion						
Absent	53	15.8	...	...	...	...
Present	113	17.0	.088	...	...	...
Pleural invasion						
Absent	115	15.8	...	...	...	...
Present	53	18.8	.393	...	...	...
Histology						
Adenocarcinoma	124	20.9	...	...	...	...
Nonadenocarcinoma	46	12.4	<.001	...	...	...
Differentiation						
Well or moderate	97	20.8	...	1	...	...
Poor	65	14.1	.002	1.810	1.194-2.743	.005
Type of surgery						
Single lobectomy	162	17.3	.152	...	...	...
Bilobectomy or pneumonectomy	8	19.5	...	...	...	...
Adjuvant therapy						
Without	134	15.9	.547	...	...	...
With	36	21.0	...	...	...	...
Postrecurrence therapy						
Without	41	7.2	...	1	...	...
With	118	21.4	.021	0.542	0.344-0.853	.008
Recurrence free interval						
≤ 24 mo	82	16.2	...	...	...	...
> 24 mo	88	18.4	.021	...	...	...
Type of recurrence						
Distant	127	15.8	...	...	...	...
Local only	43	18.8	.087	...	...	...
Number of recurrent sites						
Single	132	16.8	...	...	...	...
Multiple	38	18.6	.305	...	...	...

PRS = postrecurrence survival. See Table 1 legend for expansion of other abbreviations.

<sup>a</sup>Median age at recurrence = 66 y.

with recurrent disease characteristics, including the recurrence site, PRT, recurrence-free interval, or PS at time of recurrence, rather than with the biologically aggressive characteristics of lung cancer.

Previous studies have demonstrated the survival benefit of PRT in patients with stage I NSCLC. Nakagawa et al<sup>4</sup> and Hung et al<sup>2,3</sup> demonstrated that patients with stage I NSCLC treated either surgically or nonsurgically had a significantly better PRS than those with supportive care alone. In our study, PRT

provided a more favorable PRS than that of no treatment, similarly to previous reports. However, the results of PRS in the patients who underwent any PRT showed that surgical resection was not related to a favorable outcome. This may have been because the number of patients who received surgery for recurrent disease was too small to provide any supportive data in terms of survival benefit. However, in cases of surgical resection for recurrent lung metastasis, objective evidence supporting the role of surgery is limited because it

**Table 4—PRS Analyses in 118 Patients Who Underwent Postrecurrence Therapy**

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	Median PRS, mo	<i>P</i> Value	HR	95% CI	<i>P</i> Value
Age at recurrence, y						
< 66	63	22.4	...	...	...	...
≥ 66	55	19.5	.151	...	...	...
Sex						
Male	79	20.0	...	...	...	...
Female	39	27.2	.002	...	...	...
Smoking status						
Never smoker	43	27.6	...	...	...	...
Ever smoker	75	17.6	.035	...	...	...
Histology						
Adenocarcinoma	84	24.4	...	1	...	...
Nonadenocarcinoma	34	13.9	<.001	2.136	1.273-3.585	.004
Differentiation						
Well or moderate	66	23.1	...	...	...	...
Poor	46	18.8	.019	...	...	...
Lung metastasis						
Absent	68	19.8	...	...	...	...
Present	49	21.4	.053	...	...	...
Brain metastasis						
Absent	96	19.6	...	...	...	...
Present	21	22.6	.584	...	...	...
Bone metastasis						
Absent	100	21.9	...	1	...	...
Present	17	15.8	.001	3.288	1.783-6.062	<.001
Liver metastasis						
Absent	110	21.9	...	1	...	...
Present	7	10.5	.001	4.518	1.793-11.379	.001
Chemotherapy						
Without	15	9.6	...	1	...	...
With	103	22.7	.009	0.478	0.236-0.975	.040
Surgical resection						
Without	110	20.8	...	...	...	...
With	8	33.7	.209	...	...	...
EGFR-TKI therapy						
Without	91	17.0	...	1	...	...
With	27	41.4	.002	0.460	0.245-0.862	.015
Second line therapy						
Without	52	14.0	...	...	...	...
With	66	27.2	.004	...	...	...
Recurrence free interval						
≤ 24 mo	59	17.0	...	...	...	...
> 24 mo	59	22.4	.394	...	...	...
Type of recurrence						
Distant	85	20.8	...	...	...	...
Local only	33	21.8	.086	...	...	...
Number of recurrent sites						
Single	89	21.0	...	...	...	...
Multiple	29	20.8	.049	...	...	...

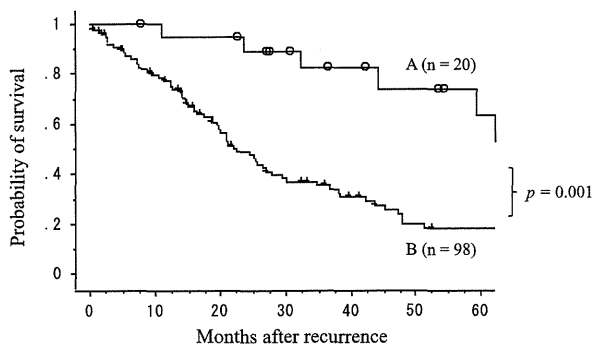
See Table 1 and 3 legends for expansion of abbreviations.

may be difficult to distinguish second primary tumors from recurrent pulmonary metastasis. Advances in genomic analysis, molecular biologic tools, or diagnostic imaging may enable more accurate diagnosis of a solitary pulmonary lesion.

Among the cohort of 118 patients with any PRT, we identified five independent favorable prognostic factors of PRS by multivariate analysis: the absence of bone or liver metastasis, chemotherapy, EGFR-TKI ther-

apy, and nonadenocarcinoma. Moreover, the result of the study showed an important aspect of a prognostic-factor based risk stratification. Median PRS times were 42.4 months for the patients lacking all five factors and 18.8 months for the patients with one of these risk factors ( $P = .001$ ).

Some authors have found that the site of initial recurrence was a prognostic factor for PRS, which agrees with the current study. Yoshino et al<sup>8</sup> demonstrated



Patients at risk of death (n = 118)

A	20	18	16	12	9	6
B	98	67	37	24	11	9

FIGURE 2. Postrecurrence survival curves of the patients lacking (A) all five unfavorable factors (not receiving epidermal growth factor receptor-tyrosine kinase inhibitor therapy and chemotherapy, liver or bone metastasis positive, nonadenocarcinoma), and (B) the patients with one of the five risk factors.

that bone metastasis was a marginally prognostic factor for PRS in patients with stage I-III NSCLC at the first resection. Assessment of bone metastatic type, osteoblastic or osteolytic, may be important as a part of post-recurrence therapeutic strategy because it has been noted that osteoblastic tumors lead to both a better prognosis and activating *EGFR* mutation presence.<sup>37</sup>

Major advances in NSCLC management have resulted from the understanding of molecular biology, development of molecule-targeting agents, and identification of biomarkers for targeted treatment. Since 2002, gefitinib has been used in Japan for the treatment of inoperable or recurrent NSCLC, and we started to administer it around the same period. It is now felt that EGFR-TKIs can improve the survival of some previously treated and untreated patients with advanced NSCLC, with the overall benefit being

driven primarily by the subgroup with *EGFR* mutations.<sup>15-17,38,39</sup> EGFR-TKIs have also improved endurance and health-related quality of life compared with platinum-based doublet chemotherapy.<sup>15-17</sup> EGFR-TKIs are, therefore, good candidates for first-line PRT in patients who have had resected adenocarcinoma with distant metastases, but only in those with *EGFR* mutations.

There are several limitations in the present study. This study is retrospective, and bias may exist. First, patient-selection bias regarding PRT was unavoidable. Curative intent therapy or systematic treatment is difficult to perform in patients with poor PS. In the current study, PS or comorbidities at the time of recurrence were not accurately evaluated. Second, distinguishing second primary tumors from recurrent pulmonary metastasis was difficult. Even if a pathologic specimen was obtained, definitive diagnosis could be difficult under the current morphology-based diagnostic criteria. Third, complete follow-up was not available for all eligible patients.

There are presently no clinical guidelines for PRT regarding resected NSCLC based on large-scale prospective studies. Molecularly targeted therapy, chemotherapeutic regimens, and surgical strategies have evolved substantially over the decades. A challenge for the future will be to create systematic treatment strategies for recurrent NSCLC according to the individual patient's recurrent-disease characteristics, including the initial recurrence site, age, sex, PS, or recurrence-free interval, and original tumor characteristics.

## CONCLUSION

This study showed that male sex, the absence of PRT, and poorly differentiated carcinoma were independent unfavorable prognostic factors of PRS in patients

Table 5—PRS of Patients With Stage I Non-small Cell Lung Cancer in Previous Series

Series/Year	Patients, No.	Incidence of Recurrence,		Type of Recurrence	Independent Favorable Factors of PRS
		No. (%)	PRS, % (y)		
Martini et al <sup>6</sup> /1995	598	159 (26.6)	NR	L/D	NR
al-Kattan et al <sup>7</sup> /1997	123	36 (29.3)	NR	L/D	NR
Nakagawa et al <sup>4</sup> /2008	397	87 (21.9)	67.7 (1) 34.4 (3)	L/D	Symptom at recurrence, negative Cervicomedastinum metastases, negative Liver metastases, negative PRT (surgery/nonsurgery)
Hung et al <sup>2</sup> /2009	933	74 (7.9)	48.7 (1) 17.6 (2)	L	PRT (surgery, chemotherapy, and/or radiotherapy)
Hung et al <sup>3</sup> /2010	933	166 (17.8)	30.2 (1) 15.1 (2)	D	Disease-free interval > 16 mo PRT
Current series/2013	919	170 (18.5)	73.5 (1) 51.4 (2)	L/D	PRT Female sex

D = distant recurrence; L = local recurrence, NR = not reported; PRT = postrecurrence therapy. See Table 3 legend for expansion of other abbreviation.

with resected stage I NSCLC. Moreover, in patients who underwent any PRT, who were receiving EGFR-TKIs and chemotherapy, and with absence of liver or bone metastasis, and with nonadenocarcinoma had a statistically significant association with favorable PRS. Further clinical studies may give more accurate information about the benefits of PRT for survival and lead to the improvement of clinical assessment and therapeutic strategies in recurrent NSCLC.

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*Dr Shimada:* contributed to the design and coordination of the study, prepared the manuscript, read and approved the final manuscript, and served as principal author.

*Dr Saji:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Yoshida:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Kakihana:* contributed to preparing the manuscript and read and approved the final manuscript.

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*Dr Nomura:* contributed to preparing the manuscript and read and approved the final manuscript.

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*Dr Kajiwara:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Ohira:* contributed to preparing the manuscript and read and approved the final manuscript.

*Dr Ikeda:* contributed to the design and coordination of the study, revised the article for important intellectual content, and read and approved the final manuscript.

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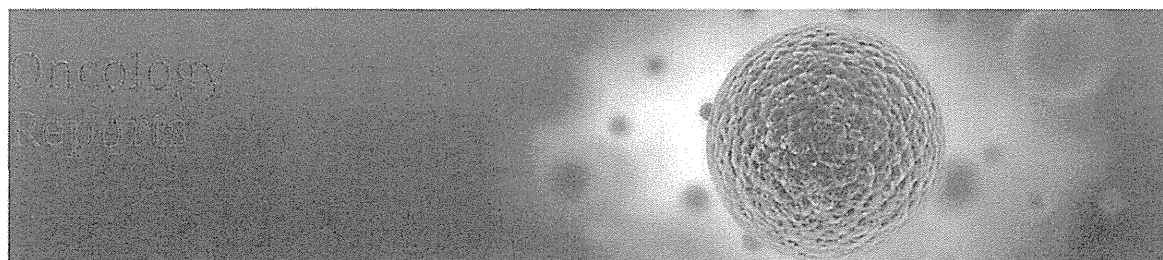
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## Gene expression profiling and molecular pathway analysis for the identification of early-stage lung adenocarcinoma patients at risk for early recurrence

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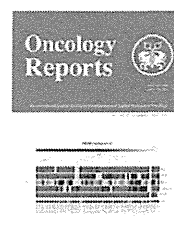
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### Abstract

Clinicohistopathological staging is insufficient to predict disease progression and clinical outcome in lung carcinoma. Based on the results of the principal component analysis of 24 samples of early-stage lung adenocarcinoma, two subgroups were identified within the early-relapse group. The histological classification of all samples of group A was poorly differentiated, whereas one out of three in group B was poorly differentiated. DAVID functional annotation analysis revealed that the molecular pathways enriched in group A included those associated with cell adhesion molecules (CAMs), cell cycle and antigen processing and presentation, whereas those in group B included CAMs, T cell receptor signaling, cytokine-cytokine receptor interaction, toll-like receptor signaling, chemokine signaling, primary immunodeficiency and natural killer cell-mediated cytotoxicity. The CAM pathway was enriched in both groups. This comprehensive gene expression and functional pathway analysis identified a distinct molecular pathway, CAMs, that correlated with the early relapse of patients with early-stage lung adenocarcinoma.

### Introduction

Lung cancer is the leading cause of cancer-related deaths in Japan and also worldwide in most developed countries. Every year, ~60,000 individuals succumb to lung cancer in Japan, and the number is increasing rapidly. Even in early-stage lung cancer, ~40% of patients with stage I and II non-small cell lung cancer (NSCLC) die from recurrent disease within 5 years despite complete resection [1,2]. The precise diagnosis and classification of cancers are critical for the selection of appropriate therapies. However, since no reliable clinical or molecular predictors are currently available, it is difficult to select high-risk patients who require more aggressive therapies such as adjuvant chemotherapy.



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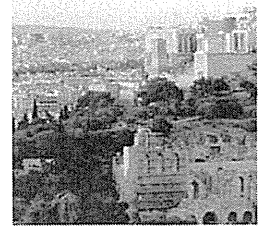
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Genetic abnormalities that exist in a certain population of early-stage lung cancer patients possibly induce aggressive phenotypes that demonstrate rapid tumor growth, persistent invasiveness and a high potential for distant metastasis. The expression of a number of genes is altered in cancer cells due to mutations, deletions, amplifications, and either the upregulation or downregulation of mRNA transcription. Comprehensive DNA microarray analysis of gene expression patterns is a powerful tool that permits the simultaneous evaluation of a large number of genes in cancer cells [3,4]. Microarray gene expression profiling has recently been used to define prognostic signatures in patients with NSCLC [5–11]. However, information concerning gene expression profiling and molecular pathways relating to the outcomes of patients with early-stage lung cancer has yet to be well characterized.



Adenocarcinoma is currently the predominant histological subtype of NSCLC. The results of several expression profiling studies have demonstrated that the expression profiles are distinctive and recapitulate the known histological subtypes [5–7]. As a significant proportion of patients relapse within 2 years, identification of early-stage patients with a poor prognosis could delineate the appropriate candidates for adjuvant therapy. The present study aimed to identify a novel prognostic signature in early-stage lung adenocarcinoma using cDNA microarray and bioinformatics analysis.

## Materials and methods

### Patient samples

Intraoperatively, immediately upon removal of a lung lobe in which a primary lung carcinoma was located, a 500-mg sample of tumor tissue was cut and immediately immersed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use, as previously reported [12]. We studied frozen specimens of lung cancer tissue from 64 randomly selected patients who underwent complete resection of stage I or II NSCLC lesions at Tokyo Medical University, Tokyo, Japan from May 2003 to December 2006. Tumor tissues were processed by the Human Tissue Bank section at our department according to standard operating procedures and protocols. Briefly, frozen tissue samples at  $-80^{\circ}\text{C}$  were pulverized, and total cellular RNA was collected from each flash-frozen sample using TRIzol RNA isolation reagent (Invitrogen). Total RNA was processed with an RNeasy Mini kit (Qiagen). *In vitro* transcription-based RNA amplification was then performed on at least 8  $\mu\text{g}$  of total RNA from each sample. The RNA quality was assessed using a bioanalyzer (model 2100, Agilent). According to the results from the RNA quality assay, 24 lung adenocarcinoma samples were selected as our dataset.

### Microarray analysis

Complementary DNA was synthesized using the T7-(IdT)24 primer: 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(IdT)24-3'. The cDNA was processed using phase-lock gel phenol/chloroform extraction (#E0032005101, Fisher). Next, *in vitro* transcriptional labeling with biotin was performed using the Enzo BioArray kit (#900182, Affymetrix). The resulting cRNA was processed again using the RNeasy Mini kit. Labeled cRNA was hybridized to an Affymetrix GeneChip (Human Genome-133 Plus 2.0 Array) according to the manufacturer's instructions. The raw fluorescence intensity data within the CEL files were preprocessed with the robust multiplex average algorithm, as implemented with the R packages from Bioconductor. This algorithm analyzes the microarray data in three steps: a background adjustment, quantile normalization, and finally summation of the probe intensities for each probe set using a log scale linear additive model for the log transform of [background corrected, normalized] PM intensities.

### Data analysis

Affymetrix Human Genome-U133 Plus 2.0 GeneChip data, quantified with MAS5, were imported into the Subio Platform (Subio Inc., Tokyo, Japan). Signals  $<1$  were replaced with 1,  $\log_2$  transformed, and then mean-subtracted by each probe set to obtain the log ratio against the average of the expression patterns. No normalization was applied.

Samples were classified into two groups, recurrence-positive and recurrence-negative. Probe sets in both groups whose detection values were absent in half of the samples were removed. At this point, 28674 out of 54682 probe sets remained. Finally, unvarying probe sets, whose log ratios were between  $-1$  and  $+1$  in all samples, were filtered out to obtain the final quality controlled probe sets [24420].

Principal component analysis (PCA) was applied to the log ratio data of quality controlled genes. We recognized that the samples in the recurrence-positive group might be distinguishable as PC1 score negative (A) and positive (B) subgroups.

We extracted the differentially expressed genes (DEGs) for both A and B subgroups. We defined DEGs for A as being  $> 4$ -fold upregulated or downregulated compared with the

average of the recurrence-negative group, and having Mann-Whitney U-test P-values of  $<0.05$  between the recurrence-negative group and the recurrence-positive A subgroup. A total of 721 probe sets were selected as DEG for A. Similarly, we obtained 274 probe sets as DEGs for B, which showed a  $>2$ -fold change and P-values of  $<0.05$  by the Mann-Whitney U-test, as compared with the recurrence-negative group.

#### Biological analysis of the DEG lists

We searched 171 and 33 enriched GO terms for DEGs determined for the A and B group, respectively, with the annotation analysis plug-in of the Subio platform [data not shown]. We further analyzed these lists with the DAVID functional annotation web tool (<http://david.abcc.ncifcrf.gov>) and obtained the lists of enriched KEGG pathways (Tables I and II).

Age	Sex	Stage	Recurrence	Outcome
62	M	IAB	-	Survived
65	F	IAB	-	Survived
68	M	IAB	-	Survived
70	F	IAB	-	Survived
72	M	IAB	-	Survived
75	F	IAB	-	Survived
78	M	IAB	-	Survived
80	F	IAB	-	Survived
82	M	IAB	-	Survived
85	F	IAB	-	Survived
88	M	IAB	-	Survived
90	F	IAB	-	Survived
92	M	IAB	-	Survived
95	F	IAB	-	Survived
98	M	IAB	-	Survived
100	F	IAB	-	Survived

**Table I**

Patient information regarding the 24 adenocarcinoma samples.

Pathway	Gene	Count	P-value
Glycolysis	PFKP	2	0.001
Glycolysis	PFKFB3	1	0.001
Glycolysis	PFKFB1	1	0.001
Glycolysis	PFKFB2	1	0.001
Glycolysis	PFKFB4	1	0.001
Glycolysis	PFKFB5	1	0.001
Glycolysis	PFKFB6	1	0.001
Glycolysis	PFKFB7	1	0.001
Glycolysis	PFKFB8	1	0.001
Glycolysis	PFKFB9	1	0.001
Glycolysis	PFKFB10	1	0.001
Glycolysis	PFKFB11	1	0.001
Glycolysis	PFKFB12	1	0.001
Glycolysis	PFKFB13	1	0.001
Glycolysis	PFKFB14	1	0.001
Glycolysis	PFKFB15	1	0.001
Glycolysis	PFKFB16	1	0.001
Glycolysis	PFKFB17	1	0.001
Glycolysis	PFKFB18	1	0.001
Glycolysis	PFKFB19	1	0.001
Glycolysis	PFKFB20	1	0.001

**Table II**

Enriched pathways in group A.

#### Ethical considerations

Written informed consent was obtained from the patients for tissue procurement prior to surgery and their medical records were maintained according to protocols approved by the Institutional Review Board of Tokyo Medical University (no. 965).

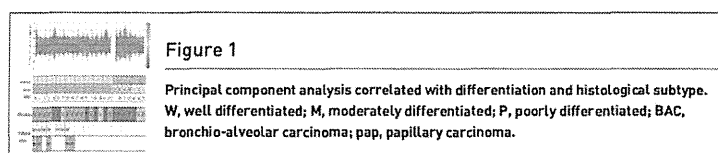
## Results

#### Patient information

As shown in Table I, there were 14 male and 10 female patients enrolled in this study. The mean age was 65.3 years [range, 42–76]. The histological classifications were all adenocarcinoma; 14 were well/moderately differentiated and 10 were poorly differentiated. The distribution of clinical staging demonstrated that most of the patients were early-stage IAB cases. Histological differentiation was significantly correlated with early recurrence ( $P=0.026$ ), whereas no significant correlations were found among pathological stages IA, IB and IIA ( $P=0.061$ ).

#### Correlation of patient outcome with putative adenocarcinoma classes

We aimed to ascertain whether lung cancer patient outcome correlates with the subclasses of lung adenocarcinomas defined herein. Based on the results of PCA of this series, two adenocarcinoma subgroups were identified within the early-relapse group of early-stage adenocarcinoma cases, which differentially expressed a broad range of gene patterns (Fig. 1).



**Figure 1**

Principal component analysis correlated with differentiation and histological subtype. W, well differentiated; M, moderately differentiated; P, poorly differentiated; BAC, bronchio-alveolar carcinoma; pap, papillary carcinoma.

Statistical analysis of the microarray data, when compared with the non-early-relapse group C, revealed 723 genes with significant differences in expression in the samples of group A, whereas 274 genes showed significant differences in expression in samples of group B. We searched 171 and 33 altered GO terms for DEGs in the A and B lists, respectively, with the annotation analysis plug-in of the Subio platform [data not shown].

The histological classification of all samples of group A was poorly differentiated, whereas only one out of three cases in group B was classified as poorly differentiated. In this series of early-stage IA-IIA adenocarcinomas, no papillary or bronchio-alveolar carcinoma subtypes were associated with recurrence within 2 years after complete resection.

#### Biological function analysis

Tables II and III document the 16 and 17 enriched pathways in groups A and B, respectively. Clusters of genes related to oncological or immunological functional signaling were found enriched in group A as were pathways such as cell adhesion molecules (CAMs), cell cycle, and antigen processing and presentation. In group B, the pathways included CAMs, T cell receptor signaling, cytokine-cytokine receptor interaction, toll-like receptor signaling, chemokine signaling pathway, primary immunodeficiency and natural killer cell mediated cytotoxicity. The CAM pathway was found to be enriched in both groups A and B.

Table III	
Enriched pathways in group B.	
Cell adhesion molecules (CAMs)	16
Cell cycle	16
Antigen processing and presentation	16
T cell receptor signaling	17
Cytokine-cytokine receptor interaction	17
Toll-like receptor signaling	17
Chemokine signaling pathway	17
Primary immunodeficiency	17
Natural killer cell mediated cytotoxicity	17

## Discussion

The development of microarray technologies has made it possible to quantitate the expression of many thousands of genes simultaneously in a given sample [3,4]. Comprehensive analysis of gene expression patterns in individual tumors should, therefore, provide detailed molecular portraits that can facilitate tumor classification. Several expression profiling studies concluded that expression profiles are distinctive and recapitulate known histological subtypes [5-7].

Genomic methods offer promise for the classification of human lung carcinomas. In one previous study, it is important to note that the performance of the adenocarcinoma classifier showed a better predictive accuracy than the squamous cell lung carcinoma (SCC) classifier (adenocarcinoma AUC = 0.83, SCC AUC = 0.68). This could have been due to the heterogeneity of the SCC samples as indicated by the two distinct subgroups showing differing clinical outcomes in this tumor type [9]. Multiple independent studies of mRNA expression profiles in lung adenocarcinoma have proven highly reproducible. Analyses of the relationship between expression profiles and tumor development and differentiation, the presence or absence of specific pathogenic mutations, patient prognosis and survival after surgical treatment, and specific histopathology all appear to be promising [13].

Adenocarcinoma is currently the predominant histological subtype of NSCLC. NSCLC composes the majority of bronchogenic carcinoma cases with a lesser fraction being small-cell lung carcinomas. The three main subtypes of NSCLC are adenocarcinoma (60%), SCC (25%) and large-cell cancer (5%). Adenocarcinoma has replaced SCC as the most frequent histological subtype over the last 25 years [1,2,14]. Therefore, we focused on adenocarcinoma of the lung, and particularly whether we could identify a novel prognostic signature of early recurrence in early-stage lung adenocarcinoma using cDNA microarray techniques.

The data indicated that patterns of gene expression obtained from cDNA microarray studies of crudely dissected lung tumors can be used to detect tumor subtypes that correlate with biological and clinical phenotypes. Specifically, patterns of gene expression were found that corresponded to the major morphological classes of lung tumors. In addition, we were able to define two subgroups of early recurrence in the adenocarcinoma cases that differed not only in gene expression patterns, but also in clinical and pathological properties, including histological differentiation and subtype. In the statistical analysis of microarray data, when compared with the non-early-recurrence group C, we revealed 723 genes with significant differences in expression in the samples of group A, whereas 274 genes showed significant differences in expression in group B. The differentially expressed genes were classified according to biological processes. We searched 171 and 33 enriched GO terms for DEGs for the A and B lists, respectively, with the annotation analysis plug-in of the Subio platform [data not shown].

Gene annotation enrichment analysis is a functional analysis technique that has gained widespread attention and for which many tools have been developed. The differentially expressed genes were classified according to biological processes and molecular functions using the functional annotation clustering tool of the DAVID bioinformatics resources. The DAVID functional clustering analysis revealed 16 significantly altered biological pathways in group A that included 3 distinct functionally related metastatic categories, specifically CAMs, cell cycle, and antigen processing and presentation. In group B, there were 17 significantly altered biological pathways, including 7 distinct functionally related metastatic categories. Notably, the CAM pathway was the most interrelated in both groups. In addition, the T cell receptor signaling pathway, cytokine-