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.[15-21] 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, mucoepidermoid 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,[22] and disease stage was determined in accordance with the *TNM Classification for Lung and Pleural Tumours*, 7th ed.[23]

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 χ^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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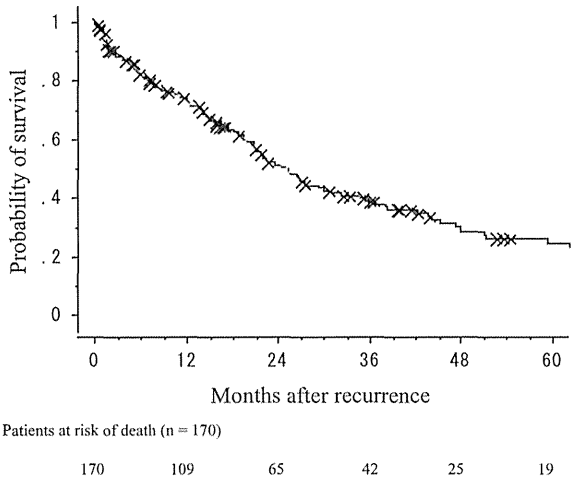

FIGURE 1. Postrecurrence survival curve of patients with non-small cell lung cancer recurrence.

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*

	Univariate Analysis			Multivariate Analysis		
Factors	Patients, No.	5-y RFP, %	*P* Value	HR	95% CI	*P* Value
Age,[a] 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.
[a]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 (≤ 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. Therefore, 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).[1-6,8,24]

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.[25-36] 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 [≤ 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,[a] 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.
[a]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[4] and Hung et al[2,3] 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*

	Univariate Analysis			Multivariate Analysis		
Factors	Patients, No.	Median PRS, mo	*P* Value	HR	95% CI	*P* 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 therapy, 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[8] demonstrated

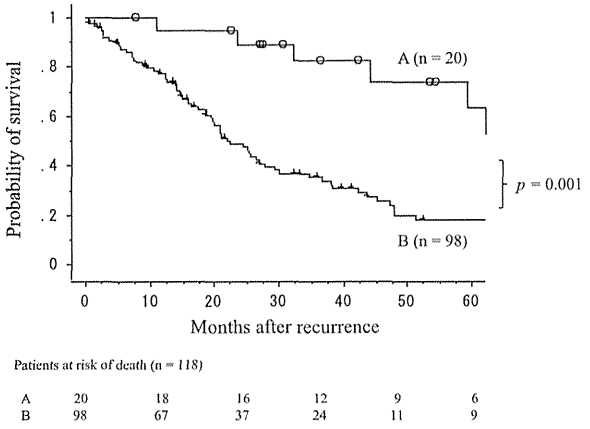

FIGURE 2. Postrecurrence survival curves of the patients lacking (A) all five unfavorable factors (not receiving epidermal growth factor receptor-tyrosine kinase inhibitor therapy and chemotherapy, liver or bone metastasis positive, nonadenocarcinoma), and (B) the patients with one of the five risk factors.

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 postrecurrence therapeutic strategy because it has been noted that osteoblastic tumors lead to both a better prognosis and activating *EGFR* mutation presence.[37]

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.[15-17,38,39] EGFR-TKIs have also improved endurance and health-related quality of life compared with platinum-based doublet chemotherapy.[15-17] 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, No. (%)	PRS, % (y)	Type of Recurrence	Independent Favorable Factors of PRS
Martini et al[6]/1995	598	159 (26.6)	NR	L/D	NR
al-Kattan et al[1]/1997	123	36 (29.3)	NR	L/D	NR
Nakagawa et al[4]/2008	397	87 (21.9)	67.7 (1)	L/D	Symptom at recurrence, negative
			34.4 (3)		Cervicomediastinum metastases, negative
					Liver metastases, negative
					PRT (surgery/nonsurgery)
Hung et al[2]/2009	933	74 (7.9)	48.7 (1)	L	PRT (surgery, chemotherapy, and/or radiotherapy)
			17.6 (2)		
Hung et al[3]/2010	933	166 (17.8)	30.2 (1)	D	Disease-free interval > 16 mo
			15.1 (2)		PRT
Current series/2013	919	170 (18.5)	73.5 (1)	L/D	PRT
			51.4 (2)		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.
Dr Honda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Nomura: contributed to preparing the manuscript 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 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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Proposal on incorporating blood vessel invasion into the T classification parts as a practical staging system for stage I non-small cell lung cancer

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ABSTRACT

Background: We investigated blood vessel invasion (BVI) as a possible negative prognostic factor in patients with stage I non-small cell lung cancer (NSCLC) according to the 7th edition of the TNM classification.
Methods: Between 1999 and 2007, a total of 694 consecutive patients with pathological stage I NSCLC underwent complete resection with systematic lymph node dissection at Tokyo Medical University Hospital. All sections of the specimens were stained by Elastica van Gieson to visualize elastic fibers and were examined to determine the prognostic symptoms of BVI. We statistically analyzed the association between BVI and clinicopathologic factors, as well as clinical outcomes.
Results: BVI was detected in 201 patients with stage I NSCLC (29.0%). The 5-year overall survival (OS) rates of the non-BVI and BVI patients were 90.5% and 66.0%, respectively ($p < 0.0001$). BVI was found to be a significant independent prognostic factor by multivariate survival analysis in stage IA and stage IB NSCLC (HR 2.591, $p < 0.001$; HR 2.347, $p = 0.009$, respectively). The 5-year OS rate of patients with BVI was significantly worse than that of patients without BVI in the T1a (94.5% vs 87.5%, $p < 0.0001$), T1b (82.7% vs 65.9%, $p < 0.0001$), and T2a (90.9% vs 61.8%, $p < 0.0001$) subgroups.
Conclusion: We identified the presence of BVI as an independent poor prognostic factor in patients with stage I NSCLC. In the future revision of the TNM staging system, the routine use of elastic fiber stains in pathological evaluations of lung cancer for BVI determination might be recommended, and tumors with BVI should be upstaged to the higher current T staging.

1. Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers and a major cause of cancer-related deaths. Pathological stage (p-stage) I NSCLC was observed in about 65% of all surgical cases [1], and these patients have the best chance of cure by surgery alone. Within the stage I designation, however, some clinicopathological characteristics may be associated with poor long-term survival. The 5-year overall survival (OS) rates in resected stage IA and IB NSCLC range from 84% to 87% and from 66% to 74%, respectively, as shown in large-scale Japanese lung cancer studies [1,2], although the 5-year OS rates were approximately more than 95% in p-stage I patients with breast cancer, colon cancer or gastric cancer.

Many studies have been reported to determine various prognostic factors other than the pathological stage, such as sex [3], age [3], smoking history [4], serum level of carcinoembryonic antigen (CEA) [5], extent of operation [3], tumor differentiation [6], tumor size [7], and number of involved lymph nodes [8]. In addition, blood vessel invasion (BVI) has been shown to be a strong independent predictor for p-stage I disease in most studies that adopted this factor as a variable for analyses [7,9–12]. Although BVI has been taken into account in the supplementary TNM staging, BVI is not a descriptor of the T component of the TNM classification. The objective of the present study was to evaluate BVI impact on survival and propose a method of incorporating BVI into T status, in relation with the 7th TNM classification.

2. Materials and methods

2.1. Patient selection

A total of 1234 consecutive patients underwent complete pulmonary resection between January 1999 and December 2007 at

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Tokyo Medical University Hospital. We defined complete resection as lobectomy or more extensive lung resection with systematic ipsilateral hilar and mediastinal lymph node dissection and with no evidence of residual cancer either macroscopically or microscopically. Of these patients, 847 were pathologically proven to have stage I NSCLC. We excluded patients who had received preoperative chemotherapy, radiotherapy or both, or who had been given a diagnosis of low-grade malignant diseases, including carcinoid, mucoepidermoid carcinoma, and adenoid cystic carcinoma. The remaining 694 patients who were pathologically confirmed to have stage I NSCLC were enrolled in this study. We also reviewed 35 patients with surgically resected pathological node-negative stage IIA NSCLC in order to compare the survival and recurrent rates.

We reviewed the medical records of each patient regarding their clinicopathologic information including age, gender, smoking history, tumor location, operation procedure, histologic type, tumor size, blood vessel invasion, lymphatic permeation, and visceral pleural invasion (VPI). Disease stages were based on the 7th edition of the TNM Classification for Lung and Pleural Tumors of the Union for International Cancer Control (UICC).

After resection, the patients were examined at 3-month intervals for 3 years, then at 6-month intervals for the next 2 years, and thereafter at 1-year intervals, in general. The evaluations included physical examination, chest roentgenogram, chest computed tomography (CT), and tumor marker measurement. Abdominal CT and brain MRI as well as bone scintigraphy were performed every year. Patients with cancer recurrence were carefully divided into 2 groups according to the site of initial relapse: locoregional or distant. The median follow-up period was 4.6 years. The Institutional Review Board of our hospital approved the protocols for data collection and analyses, and waived the need to obtain written informed consent from each patient.

2.2. Histopathologic studies

Histopathologic studies were performed according to World Health Organization criteria. After fixing the specimens in 10% formalin and embedding them in paraffin, sections were stained with hematoxylin and eosin and by Elastica van Gieson (EvG) staining to visualize elastic fibers. Detailed examinations of BVI were routinely performed at our institution. Blood vessels were identified by the presence of erythrocytes in the lumen or an endothelial cell lining or the presence of elastic tissue around larger vessels. Sections stained by EvG were examined for the presence of BVI. The presence of BVI was determined by identifying conspicuous clusters of intravascular cancer surrounded by an elastic fiber layer (Fig. 1). On the other hand, lymphatic permeation was determined to be present when tumor cells floating in lymphatic vessels with no supporting smooth muscles or elastic fibers were identified. We confirmed that lumens within the bronchovascular bundle, subpleural, and intralobular pleural space were lymphatic vessels by immunostaining with anti-D2-40 antibody.

2.3. Statistical analysis

Overall survival (OS) and recurrence-free survival (RFS) were estimated using the Kaplan–Meier method, and differences in survival rates were determined by log-rank analysis. OS was defined as the time elapsed from the date of pulmonary resection to the date of death. RFS was defined as the time elapsed from the date of pulmonary resection to the date of the first recurrence or last follow-up showing no recurrence. The last follow-up observation was censored if the patient was alive or lost to follow-up. Univariate analysis was conducted among the different groups. Categorical variables were analyzed using the chi-square test. Differences between 2 groups were tested using the Mann–Whitney *U* test. Multivariate analysis was performed by the Cox proportional hazards model using the significant factors identified from univariate analysis to examine the association between survival and potential prognostic factors. All *p*-values were two-sided and *p*-values of <0.05 were considered to indicate a statistically significant difference. All statistical calculations were performed using StatView for Windows version 5.0 (SAS Institute Inc., Cary, NC, USA).

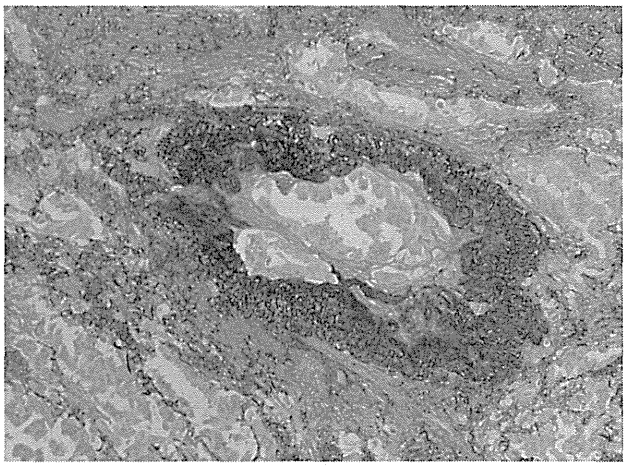

Fig. 1. Elastica van Gieson staining of a tumor with blood vessel invasion (BVI). The presence of BVI was determined by identifying conspicuous clusters of intravascular cancer surrounded by an elastic layer (original magnification, 400×).

3. Results

The characteristics of the patients are shown in Table 1a. BVI was detected in 201 patients (29.0%). The 5-year OS rates of non-BVI and BVI patients were 90.5% and 66.0%, respectively (Fig. 2). The relationship between clinicopathological prognostic factors and BVI is shown in Table 1b. BVI prevalence was significantly higher in men, ever smokers, non-adenocarcinoma, positive lymphatic permeation, positive visceral pleural invasion, and p-stage IB ($p < 0.05$). Tumor size was significantly larger in BVI tumors than in non-BVI tumors ($p < 0.0001$).

In patients with p-stage IA tumors, men, over 70 years old, ever smokers, tumor size over 2 cm in diameter, non-adenocarcinoma, and the presence of BVI were statistically significant poor prognostic factors. Multivariate survival analysis showed that a tumor size over 2 cm in diameter and the presence of BVI were statistically significant independent prognostic factors, as well as ages over 70 years old and non-adenocarcinoma (hazard ratio [HR] = 2.096, $p = 0.0040$; HR = 2.591, $p = 0.0004$, respectively)

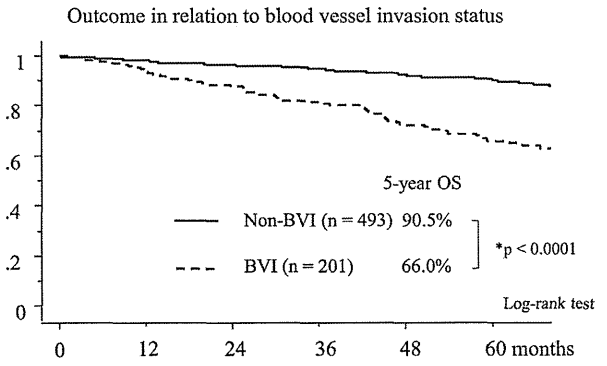

Fig. 2. Survival curves for blood vessel invasion (BVI) and non-BVI. *$p < 0.05$.

Table 1a
Patient characteristics (stage I, $n = 694$; stage IIA, $n = 35$).

Variable	Number (%)				p value (stage IA vs stage IB)
	All ($n = 694$)	Stage IA ($n = 423$)	Stage IB ($n = 271$)	Stage IIA (T2bN0) ($n = 35$)	
Sex					<0.0001*
Men	371 (53.5)	200 (47.3)	171 (63.1)	32 (91.4)	
Women	323 (46.5)	223 (52.7)	100 (36.9)	3 (8.6)	
Median age (range)	66.0 (22–86)	65.0 (22–86)	67.0 (38–86)	69.0 (48–86)	0.0381*
Smoking history					0.0030*
Never smoker/unknown	315 (45.4)	211 (49.9)	104 (38.4)	5 (14.3)	
Ever smoker	379 (54.6)	212 (50.1)	167 (61.6)	30 (85.7)	
Tumor location					0.9999
Right	451 (65.0)	275 (65.0)	176 (64.9)	25 (71.4)	
Left	243 (35.0)	148 (35.0)	95 (35.1)	10 (28.6)	
Operation procedure					0.5355
Lobectomy	669 (96.4)	406 (96.0)	263 (97.0)	33 (94.3)	
Bilobectomy	25 (3.6)	17 (4.0)	8 (3.0)	2 (5.7)	
Histological type					0.0002*
Adenocarcinoma	568 (81.8)	365 (86.3)	203 (74.9)	18 (51.4)	(Ad vs non-Ad)
Squamous cell carcinoma	97 (14.0)	47 (11.1)	50 (18.4)	15 (42.9)	
Large cell carcinoma	22 (3.2)	8 (1.9)	14 (5.2)	2 (5.7)	
Others	7 (1.0)	3 (0.7)	4 (1.5)	0 (0)	
Median tumor size (cm) (range)	2.3 (0.4–5.0)	1.9 (0.4–3.0)	3.5 (0.6–5.0)	6.0 (5.1–7)	<0.0001*
BVI	201 (29.0)	73 (17.2)	128 (47.2)	13 (37.1)	<0.0001*
Lymphatic permeation	237 (34.1)	101 (23.8)	136 (50.2)	17 (48.6)	<0.0001*
VPI	122 (17.6)	0 (0)	122 (45.0)	13 (37.1)	–
pT factor					–
T1a	263 (37.9)	263 (62.2)	0 (0)		
T1b	160 (23.1)	160 (37.8)	0 (0)		
T2a	271 (39.0)	0 (0)	271 (100)		

Ad, adenocarcinoma; non-Ad, other histological types; BVI, blood vessel invasion; VPI, visceral pleural invasion.
* $p < 0.05$.

Table 1b
Patient characteristics in the 2 groups according to clinicopathologic factors ($n = 694$).

Variable	Number (%)		p-Value [non-BVI vs BVI]
	Non-BVI ($n = 493$)	BVI ($n = 201$)	
Sex			<0.0001*
Men	228 (46.2)	143 (71.1)	
Women	265 (53.8)	58 (28.9)	
Age			0.0705
≤70 years old	143 (29.0)	73 (36.3)	
>70 years old	350 (71.0)	128 (63.7)	
Smoking history			<0.0001*
Ever Smoker	229 (46.5)	150 (74.6)	
Never smoker/unknown	264 (53.5)	51 (25.4)	
Tumor location			0.9302
Right	321 (65.1)	130 (64.7)	
Left	172 (34.9)	71 (35.3)	
Histological type			<0.0001*
Ad	434 (88.0)	134 (66.7)	
Non-Ad	59 (12.0)	67 (33.3)	
Operation procedure			0.8225
Lobectomy	476 (96.6)	193 (96.0)	
Bilobectomy	17 (3.4)	8 (4.0)	
Median tumor size (cm) (range)	2.0 (0.4–5.0)	3.0 (0.7–5.0)	<0.0001*
Lymphatic permeation			<0.0001*
present	101 (20.5)	136 (67.7)	
absent	392 (79.5)	65 (32.3)	
VPI			<0.0001*
present	52 (10.5)	70 (34.8)	
absent	441 (89.5)	131 (65.2)	
p-stage			<0.0001*
IA	350 (71.0)	73 (36.3)	
IB	143 (29.0)	128 (63.7)	

Non-BVI, without blood vessel invasion; BVI, with blood vessel invasion; Ad, adenocarcinoma; non-Ad, other histological types; VPI, visceral pleural invasion.
* $p < 0.05$.

(Table 2a). In patients with p-stage IB tumors, men, ever smokers, non-adenocarcinoma, the presence of BVI, and the presence of lymphatic permeation were significant poor prognostic factors. Multivariate survival analysis showed that the presence of BVI was a significant independent prognostic factor, as well as men and the presence of lymphatic permeation (HR = 2.347, $p = 0.0088$) (Table 2b).

Thus, we analyzed the OS of p-stage IA patients stratified by tumor size (≤2 cm, T1a; >2 cm, T1b) and BVI status (presence or absence) (Fig. 3a), and we also analyzed the OS of p-stage IB patients stratified by BVI status (Fig. 3b).

In the p-stage IA patient cohort, subgroup analysis of the patients revealed 5-year OS rates of 94.5%, 87.1%, 82.7%, and 65.9% for patients with T1a/non-BVI, T1a/BVI, T1b/non-BVI, and T1b/BVI, respectively (Fig. 3a). The differences in survival were statistically significant between patients with T1a/BVI and T1a/non-BVI tumors, and between patients with T1b/BVI and T1b/non-BVI tumors ($p < 0.001$ and $p = 0.034$, respectively). There was no significant difference in the survival rates between the T1a/BVI and T1b/non-BVI subgroups ($p = 0.2604$). On the other hand, in the p-stage IB patient cohort, subgroup analysis of the patients revealed 5-year OS rates of 90.3% and 61.5% for patients with T2a/non-BVI and T2a/BVI tumors, respectively, with statistically significant difference ($p < 0.001$) (Fig. 3b).

We also analyzed the prognosis of T1b, T2a and T2b tumors (Table 3). There was no significant difference in survival between the patients with T1b/non-BVI and T2a/non-BVI tumors ($p = 0.0753$). There was no significant difference in survival between patients with T1b/BVI and T2a/BVI tumors, and between patients with T2a/BVI and T2b tumors ($p = 0.7364$ and $p = 0.2394$, respectively).

The patients with T1a/BVI tumors had lower RFS than the patients with T1b/non-BVI, with no significant difference ($p = 0.2090$) (Fig. 3c). However, in RFS curves, similar relationships to OS curves were observed among these subgroups with each pathological stage tumor (Fig. 3c and d).

Table 2a
Univariate and multivariate analyses of prognostic factors in stage IA patients.

Variable	UVA	MVA		
	p-Value	Hazard ratio	95%CI	*p*-Value
Sex: men (vs women)	0.0159*	1.205	0.5921–2.451	0.6076
Age: >70 (vs ≤70)	0.0125*	1.852	1.091–3.145	0.0226*
Smoking history: ever smoker (vs never smoker)	0.0059*	1.290	0.601–2.770	0.5134
Operation procedure: bilobectomy (vs lobectomy)	NS (0.1968)		Not included in MVA	
Tumor location	NS (0.387)		Not included in MVA	
Tumor size: >2 cm (vs ≤2 cm)	0.0007*	2.096	1.266–3.472	0.0040*
Histologic type: non-Ad (vs Ad)	<0.0001*	2.899	1.624–5.176	0.0003*
BVI: present (vs absent)	0.0001*	2.591	1.529–4.386	0.0004*
Lymphatic permeation: present (vs absent)	NS (0.4553)		Not included in MVA	

UVA, univariate analysis; MVA, multivariate analysis; Ad, adenocarcinoma; non-Ad, other histological types; BVI, blood vessel invasion.
* $p < 0.05$.

Table 2b
Univariate and multivariate analyses of prognostic factors in stage IB patients.

Variable	UVA	MVA		
	p-Value	Hazard ratio	95%CI	*p*-Value
Sex: men (vs women)	<0.0001*	3.690	1.475–9.259	0.0052*
Age: >70 (vs≤70)	NS (0.7117)		Not included in MVA	
Smoking history: ever smoker (vs never smoker)	0.0007*	1.016	0.475–2.037	0.9657
Operation procedure: bilobectomy (vs lobectomy)	NS (0.5782)		Not included in MVA	
Tumor location	NS (0.6533)		Not included in MVA	
Tumor size: >3 cm (vs≤3 cm)	NS (0.0665)		Not included in MVA	
Histologic type: non-Ad (vsAd)	0.0006*	1.226	0.716–2.101	0.4575
BVI: present (vs absent)	<0.0001*	2.347	1.239–4.464	0.0088*
Lymphatic permeation: present (vs absent)	<0.0001*	2.288	1.279–4.082	0.0053*
VPI: present (vs absent)	NS (0.8943)		Not included in MVA	

UVA, univariate analysis; MVA, multivariate analysis; Ad, adenocarcinoma; non-Ad, other histological types; BVI, blood vessel invasion; VPI, visceral pleural invasion.
* $p < 0.05$.

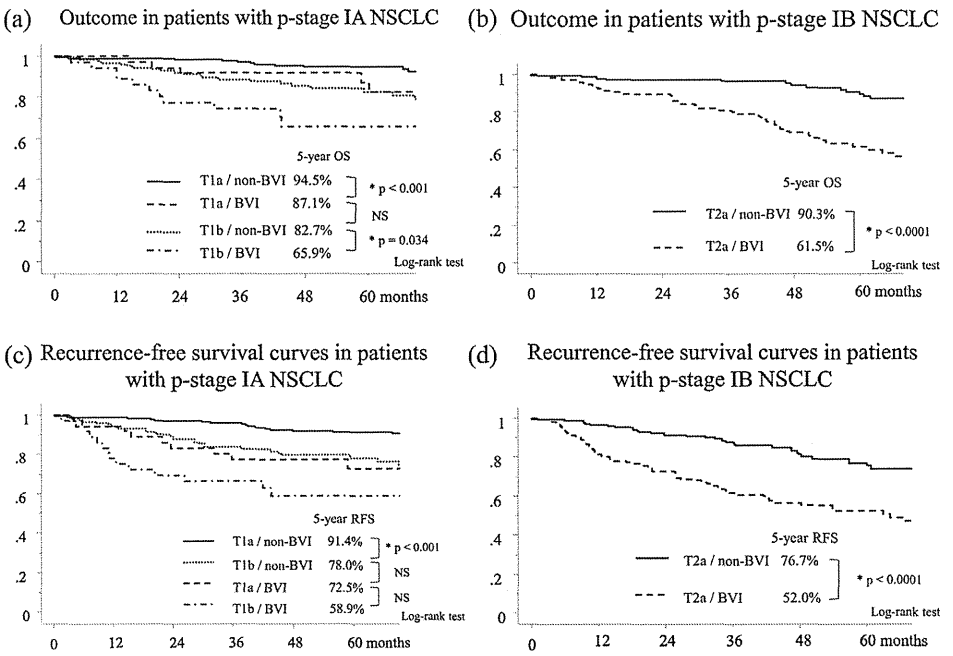

Fig. 3. (a) Survival curves and 5-year overall survival (OS) rates stratified by T-factor (T1a or T1b) and blood vessel invasion (BVI) status in patients with p-stage IA non-small cell lung cancer (NSCLC). (b) Survival curves and 5-year OS rates stratified by BVI status in patients with p-stage IB NSCLC. (c) Recurrence-free survival (RFS) curves and 5-year RFS rates stratified by T-factor (T1a or T1b) and blood vessel invasion (BVI) status in patients with p-stage IA non-small cell lung cancer (NSCLC). (d) RFS curves and 5-year RFS rates stratified by BVI status in patients with p-stage IB NSCLC. *$p < 0.05$.

Table 3
Proposal on incorporating vascular invasion into T classification.

Group	n	5yOS	p-value	7th Edition stage (N0M0)	Our proposal stage (N0M0)
T1a/non-BVI	227	94.5%	< 0.0001	IA	IA
T1a/BVI	38	87.5 %	NS (0.2604)	IA	**IB**
T1b/non-BVI	125	82.7%	0.034	IA	**IB**
T1b/BVI	39	65.9%	0.0002 NS (0.0753)	IA	**IIA**
T2a/non-BVI	145	90.9%	< 0.0001 NS (0.7364)	IB	IB
T2a/BVI	130	61.8%	NS (0.2394)	IB	**IIA**
T2b	36	68.7%		IIA	IIA

T-factor and stage in bold in our proposal differ from those in the 7th edition classification. Non-BVI, without blood vessel invasion; BVI, with blood vessel invasion; 5y OS, 5-year overall survival rate.

4. Discussion

The TNM stage classification was developed as a benchmark for prognosis and treatment options. For patients with stage I lung cancer, however, survival outcomes vary, thus stage I NSCLC comprises a heterogeneous group with different prognoses [13]. BVI has been shown to be a strong independent predictor for p-stage I disease as a variable for analyses [7,9–12,14], with only few exceptions [15,16]. In several other malignancies, BVI has similarly been shown to predict poor outcome [17–19], and its value has been commonly recognized to the point that it is included in the AJCC staging system for testicular cancer [20]. The first studies about the prognostic role of BVI in lung cancer date back to the late 1950s [21]. Despite the numerous reports of BVI as a negative prognostic factor and that BVI has been taken into account in the supplementary TNM staging, BVI has not yet been incorporated into the T classification component.

One possible reason may be that there is a lack of standardization of evaluating BVI. Many variations regarding the method of BVI assessment exist, although in most studies BVI was defined as the presence of neoplastic structures inside the lumen of a vessel. Ichinose et al. reported that venous invasion was one of the significant prognostic factors among patients with completely resected NSCLC [22]. On the other hand, arterial invasion was reported to be strongly associated with 5-year survival in patients with stage I NSCLC [23]. Arterial and venous invasion has not yet been studied pathologically separately in our institute, because discrimination was not always possible. Some studies evaluated BVI by staining with hematoxylin and eosin alone or in combination with EvG stain or by staining with Victoria blue hematoxylin and eosin, which can lead to significant heterogeneity. The reported detection rates of BVI in pathological stage I NSCLC case without any elastic fiber stains were 11–17% [15,24]. In contrast, those with elastic fiber stains ranged from 21% to 56% and were higher than those without [9,16,25]. We uniformly used hematoxylin and eosin and EvG stains on all tumors and the detection rate was 28.9% for patients with pathological stage I, which was similar to previous studies [9,16,25]. These findings suggest that elastic fiber staining helps pathologists to identify BVI more accurately in almost all cases. In the latest 7th edition of the TNM classification, VPI is clearly defined and T1 tumors remain to be upgraded to T2a when the visceral pleural elastic layer is invaded. Elastic fiber staining is also helpful in identifying VPI. We therefore recommend the routine use of elastic fiber stains in pathological evaluations of lung cancer, not only for VPI determination, as recommended in the TNM classification, but also for BVI determination, especially in patients with stage I NSCLC, to reflect more accurate, prognosis.

In this series, we showed that the 5-year OS rate of BVI patients was significantly lower than that of non-BVI patients (Table 3). These results indicate that T1a tumors with BVI should be classified as T1b, T1b tumors with BVI as T2a, and T2a tumors with BVI as T2b. This would also be consistent with other tumors with BVI being upgraded to the next T level. Our proposal on incorporating BVI into T classification can be framed to yield a better staging system for stage I NSCLC.

BVI is one of the steps leading to metastatic diffusion, and this may be the reason that BVI is associated with a poorer prognosis. Tumor cells from the primary neoplasm may penetrate these new vessels and escape from the primary site to distant organs. The relationship between tumor vessels, intravascular tumor cell invasion, and metastases has been studied in animal models [26]. In our series, there were 44 patients and 74 with recurrence in stage IA and stage IB, respectively. Among these patients, the patterns of initial recurrence included distant metastasis in 31 patients (70.5%) and in 58 (78.3%), respectively (data not shown). Classified by BVI status, recurrence developed in 17 (23.3%) of stage IA patients with BVI, and recurrence in 27 (7.7%) of these patients with non-BVI, with significant difference ($p = 0.0004$, data not shown). However, there were high rates of distant metastasis in both groups, and there were no significant differences in initial recurrence patterns. In the stage IB patients, recurrence developed in 51 (39.8%) of patients with BVI, and recurrence in 23 (16.1%) of patients with non-BVI, with a significant difference ($p < 0.0001$, data not shown). However, there were high rates of distant metastasis in both groups, and there were no significant differences in initial recurrence patterns. Surgery is considered to be the standard treatment for early-stage NSCLC. However, distant metastasis occurred in nearly 60–70% of recurrence patients with stage I NSCLC after complete resection [27,28]. Micrometastasis of the tumor is generally regarded as the cause of recurrence; therefore systemic chemotherapy after surgery is a rational strategy to reduce the risk of recurrence and metastasis.

Recent randomized controlled trials have demonstrated the survival benefit varied with stage and the usefulness of platinum-based adjuvant chemotherapy in p-stage II to IIIA NSCLC patients [29–31]. For stage IB adenocarcinoma patients, based on a large adjuvant trial on oral uracil-tegafur (UFT), UFT adjuvant

chemotherapy is recommended as the standard treatment in Japan [32]. Although surgery alone remains the standard treatment for patients with stage IA disease, recent Japanese studies also showed that oral UFT may improve survival in patients with p-stage IA showing a tumor size of 2–3 cm [32,33]. Recently, the proposed IASLC/ATS/ERS classification of lung adenocarcinoma identified histological categories with prognostic differences that may be helpful in identifying candidates for adjuvant therapy and was associated with BVI [34,35], but there might be some lack of preparation to incorporate the classification into the staging system. In the present study, when we divided the study population stratified by BVI, the patients with BVI have worse survival than those without BVI (Table 3). This classification can incorporate the prognostic impact of BVI status into the 7th edition T classification reasonably well, and patients with each stage of NSCLC with BVI may therefore be good candidates for adjuvant chemotherapy. BVI is an important parameter to venture postoperative poor prognostic groups in a strategical staging system.

Due to the retrospective analysis in a single institute, which evaluated cases from 1999, and due to a small sample size compared with T2b patients, making it impossible to draw any statistically significant conclusions in these subgroups, our proposal is not complete. Furthermore, it is difficult to evaluate BVI in the clinical staging setting. However, our data clearly indicate that BVI has a very strong prognostic impact. Prospective multi-institutional studies are mandatory to further validate the prognosis of BVI in resected stage I NSCLC.

In conclusion, despite the limitations mentioned above, we have demonstrated the prognostic power of BVI as a single independent pathologic marker for NSCLC, and our results have indicated that T1a tumors with BVI should, unlike in the 7th TNM classification, be classified as T1b, T1b tumors with BVI as T2a, and T2a tumors with BVI as T2b. In future revisions of the TNM staging system, we recommend the routine use of elastic tissue stains in pathological evaluations of lung cancer for BVI determination, and we believe that tumors with BVI should be upstaged to the above T stages. As this will affect staging criteria, additional studies employing standard methodology to assess BVI are needed to further clarify the underlying reasons why tumors with BVI have an unfavorable prognosis.

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Conflict of interest statement

None declared.

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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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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.

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°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

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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˚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 μg 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-(dT)24 primer: 59-GGCCAGTGAATTGTA ATACGACTCACTATAGGGAGGCGG-(dT)24-39. 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 multichip 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, log2 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).

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).

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 I. Patient information regarding the 24 adenocarcinoma samples.

Variables	Total samples	Early recurrence		P-value
		Positive (n=8)	Negative (n=16)	
Median age (range)	65.3 (42-76)	67.0 (55-76)	64.5 (42-76)	0.551
Gender				0.143
Male	14	3	11	
Female	10	5	5	
Histological differentiation				0.026[a]
Well/moderate	14	2	11	
Poor	10	6	4	
p-Stage				0.061
IA	14	2	12	
IB	8	5	3	
IIA	2	1	1	

[a]Significant difference.

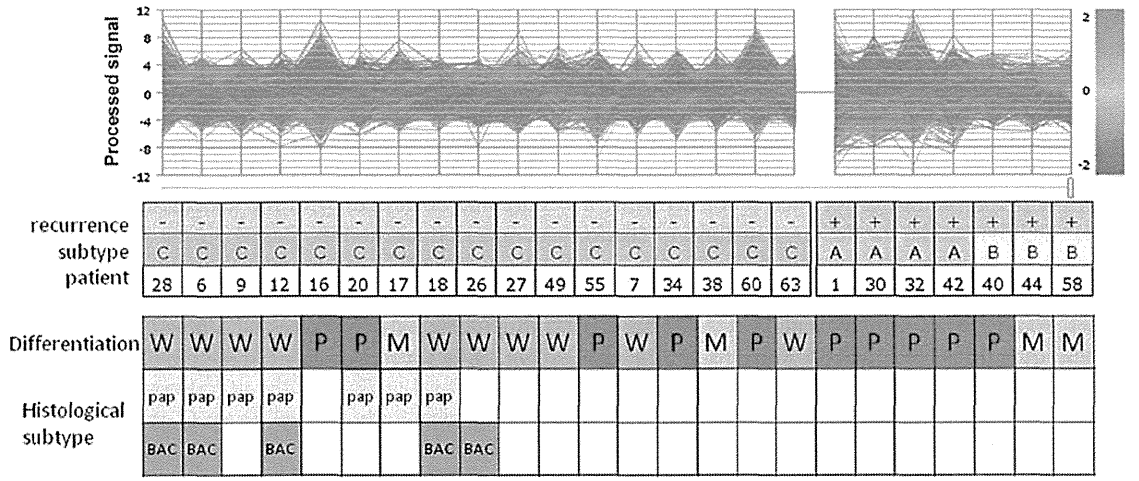

Figure 1. Principal component analysis correlated with differentiation and histological subtype. W, well differentiated; M, moderately differentiated; P, poorly differentiated; BAC, bronchio-alveolar carcinoma; pap, papillary carcinoma.

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,

Table II. Enriched pathways in group A.

Category	Term	Count	%	P-value	Benjamini
KEGG_PATHWAY	Asthma	8	1.5	0.000019	0.0024
KEGG_PATHWAY	Allograft rejection	8	1.5	0.000085	0.0053
KEGG_PATHWAY	Graft-versus-host disease	8	1.5	0.00014	0.0061
KEGG_PATHWAY	Cell adhesion molecules (CAMs)	14	2.5	0.00017	0.0052
KEGG_PATHWAY	Type I diabetes mellitus	8	1.5	0.00023	0.0059
KEGG_PATHWAY	Intestinal immune network for IgA production	8	1.5	0.00062	0.013
KEGG_PATHWAY	Autoimmune thyroid disease	8	1.5	0.0008	0.014
KEGG_PATHWAY	Hematopoietic cell lineage	10	1.8	0.0011	0.017
KEGG_PATHWAY	Systemic lupus erythematosus	10	1.8	0.003	0.041
KEGG_PATHWAY	Antigen processing and presentation	8	1.5	0.013	0.15
KEGG_PATHWAY	PPAR signaling pathway	7	1.3	0.018	0.19
KEGG_PATHWAY	Oocyte meiosis	9	1.6	0.018	0.18
KEGG_PATHWAY	Viral myocarditis	7	1.3	0.02	0.18
KEGG_PATHWAY	Arachidonic acid metabolism	6	1.1	0.027	0.22
KEGG_PATHWAY	Cell cycle	9	1.6	0.036	0.27
KEGG_PATHWAY	Drug metabolism	6	1.1	0.04	0.27

Table III. Enriched pathways in group B.

Category	Term	Count	%	P-value	Benjamini
KEGG_PATHWAY	T cell receptor signaling pathway	11	4.8	0.0000046	0.00048
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	16	7	0.0000075	0.00039
KEGG_PATHWAY	Toll-like receptor signaling pathway	9	3.9	0.00014	0.0047
KEGG_PATHWAY	Cell adhesion molecules (CAMs)	10	4.3	0.00016	0.0042
KEGG_PATHWAY	Leukocyte transendothelial migration	8	3.5	0.0021	0.041
KEGG_PATHWAY	Chemokine signaling pathway	10	4.3	0.0021	0.035
KEGG_PATHWAY	Intestinal immune network for IgA production	5	2.2	0.0064	0.091
KEGG_PATHWAY	Autoimmune thyroid disease	5	2.2	0.0074	0.091
KEGG_PATHWAY	Primary immunodeficiency	4	1.7	0.016	0.17
KEGG_PATHWAY	Type I diabetes mellitus	4	1.7	0.026	0.24
KEGG_PATHWAY	Axon guidance	6	2.6	0.047	0.36
KEGG_PATHWAY	Cytosolic DNA-sensing pathway	4	1.7	0.052	0.37
KEGG_PATHWAY	Natural killer cell mediated cytotoxicity	6	2.6	0.053	0.35
KEGG_PATHWAY	NOD-like receptor signaling pathway	4	1.7	0.069	0.41
KEGG_PATHWAY	Focal adhesion	7	3	0.087	0.46
KEGG_PATHWAY	RIG-I-like receptor signaling pathway	4	1.7	0.095	0.47
KEGG_PATHWAY	Prion diseases	3	1.3	0.1	0.47

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-cytokine receptor interaction, toll-like receptor signaling pathway, chemokine signaling pathway, primary immunodeficiency and natural killer cell mediated cytotoxicity were also altered (Tables II and III). These results suggest that the possibility of metastasis of early-stage lung adenocarcinoma was closely related to the CAM pathway. Interestingly, considering the relationship between group A or group B and histological differentiation as poor or well/moderate, respectively, the metastatic possibility of poorly differentiated early adenocarcinoma appeared to be correlated with tumor development factors, such as the cell cycle, whereas that of well/moderately differentiated early-stage adenocarcinoma appeared to be correlated with host immunological factors, such as the T cell receptor signaling pathway, cytokine-cytokine receptor interaction, the toll-like receptor signaling pathway, the chemokine signaling pathway, primary immunodeficiency and natural killer cell mediated cytotoxicity.

Our results suggest that the particular genes that define the clusters and molecular pathways, or that are associated with early recurrence, likely reflect the characteristics of the particular tumors included in the analysis. Current therapy for patients with early-stage disease usually consists of surgical resection without adjuvant treatment. Clearly, the identification of a high-risk group among early-stage patients would lead to consideration of additional therapeutic interventions, possibly leading to improved survival of these patients.

To our knowledge, this is the first study utilizing cDNA microarray techniques, followed by molecular functional pathway analysis, concerning the early recurrence of early-stage adenocarcinoma of the lung. However, there were some limitations to this study. Firstly, this was a small data set analysis at a single institute. A large cohort sample of patients from multiple institutions is needed. Secondly, the potential interactions of the many specific individual genes and their clusters in lung tumor biology and clinical outcome exist. This may be due to the different platforms used (different genes analyzed) and the different algorithms for selecting functional categories. Thirdly, hierarchical clustering methods and functional analysis offer a powerful approach to class discovery, but provide no means of determining validity for the classes discovered. This is still a putative functional analysis. It is important to state that several *in vitro* and *in vivo* studies are still needed to demonstrate whether these mechanisms are effective in reality.

In conclusion, in the present study, we present a comprehensive gene expression analysis and functional pathway analysis of early-stage lung adenocarcinomas, wherein we identified a distinct molecular pathway category, the CAMs, which correlated with the early relapse of early-stage lung adenocarcinoma subclasses. Further *in vitro* and *in vivo* studies, which can demonstrate these mechanisms, are warranted.

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