Table 1. Univariate Analysis of Overall Survival Rate of Patients Stratified by Smoking Extent

Group	Pack-Years (PY)	Number of Patients	Overall Survival Rate at 5 Years (%)	p Value by Log-Rank Test		
A	PY = 0	528	85.2	vs	Group B	0.562
					Group C	0.837
					Group D	0.002[a]
					Group E	<0.001[a]
					Group F	0.002[a]
B	0 < PY ≤ 10	75	89.2	vs	Group C	0.698
					Group D	0.034[a]
					Group E	0.009[a]
					Group F	0.015[a]
C	10 < PY ≤ 20	74	84.5	vs	Group D	0.049[a]
					Group E	0.014[a]
					Group F	0.021[a]
D	20 < PY ≤ 40	182	73	vs	Group E	0.421
					Group F	0.527
E	40 < PY ≤ 60	126	69.7	vs	Group F	0.996
F	PY > 60	85	69.3	...	...	...

[a] Indicates significance.

60) were 85.2%, 89.2%, 84.5%, 73.0%, 69.7%, and 69.3%, respectively. The differences in overall survival between groups A and D, groups B and D, groups C and D, groups A and E, groups B and E, groups C and E, groups A and F, groups B and F, and groups C and F were significant, whereas those between groups A and B, groups A and C, groups B and C, groups D and E, groups D and F, and groups E and F were insignificant (Table 1). Data were therefore optimally separated into groups A, B, and C ($0 \leq PY \leq 20$) and groups D, E, and F ($PY > 20$) for future analysis (Fig 1B).

Table 2 presents the overall 5-year survival rates for different clinical features. Univariate analysis identified four significant unfavorable prognostic factors: age, sex,

Table 2. Clinical Characteristics and Outcomes

Characteristics	Number of Patients (%)	5-Year Overall Survival Rate (%)	Univariate p Value	Multivariate Analysis		
				HR	95% CI	p Value
Total	1,070 (100)	80.3				
Age (y)						
<65	562 (53)	83.3	0.002[a]	1		
>65	508 (47)	76.9		1.36	1.051–1.760	0.02[a]
Sex						
Women	571 (53)	85.4	<0.001[a]	1		
Men	499 (47)	74.4		1.022	0.723–1.443	0.904
Smoking history						
0 ≤ PY ≤ 20	677 (63)	85.5	<0.001[a]	1		
PY > 20	393 (37)	71.1		1.826	1.296–2.572	0.001[a]
Tumor size on chest CT (cm)						
< 2.0 (T1a)	703 (66)	85.2	<0.001[a]	1		
2.1–3.0 (T1b)	367 (34)	71.9		1.824	1.412–2.356	<0.001[a]
Tumor laterality						
Right	675 (63)	80.9	0.465	Not included in multivariable model		
Left	395 (37)	79.5				
Primary lobe						
Upper or middle lobe	719 (67)	81.2	0.484	Not included in multivariable model		
Lower lobe	351 (33)	78.7				

[a] Indicates significance.

CI = confidence interval; CT = computed tomography; HR = hazard ratio for death; PY = pack-years.

smoking history, and tumor size measured from the chest computed tomography at diagnosis. According to multivariate analysis, old age, higher PY in the smoking history, and a larger tumor remained significant independent prognostic factors.

Pathological Prognostic Factors

After examining postoperative pathological factors in the patients, seven significant unfavorable prognostic factors were identified from univariate analysis: size of the resected tumor, histologic differentiation, lymphatic permeation, intratumoral vascular invasion, visceral pleural invasion, N status, and intratumoral metastasis (Table 3). Multivariate analysis identified moderate or poor histologic differentiation, lymphatic permeation, intratumoral vascular invasion, visceral pleural invasion, and lymph node metastasis as significant independent prognostic factors.

Correlation Between Smoking History and Pathological Characteristics

Correlations between smoking history and pathological characteristics are displayed in Table 4. Patients with greater than 20 PY smoking history had significantly high numbers of moderately or poorly differentiated carcinomas with lymphatic permeation, intratumoral vascular invasion, pleural invasion, and lymph node metastases.

To offset the prognostic impact of comorbidities associated with cigarette smoking, we investigated recurrence-free proportions in addition to overall survival rates. The 5-year recurrence-free proportion for patients with greater than 20 PY smoking history was significantly lower than for patients with 20 or less PY smoking history (71.0% and 80.5%, respectively; $p = 0.002$; Fig 2).

Overall Survival Rate, Recurrence-Free Proportion, and Correlation Between Smoking History and Pathological Characteristics in Patients With Pathological Stage IA Lung Adenocarcinoma

Of the 1,070 patients with clinical stage IA lung adenocarcinoma, 691 (65%) were also diagnosed with pathological stage IA lung adenocarcinoma. The overall 5-year survival rates of these patients with 20 or less PY smoking history and greater than 20 PY smoking history were 96.5% and 85.9%, respectively ($p < 0.001$; Fig 3).

Correlations between smoking history and pathological characteristics for patients with pathological stage IA tumors are displayed in Table 5. Patients with greater than 20 PY smoking history had significantly higher numbers of moderately or poorly differentiated carcinomas or carcinomas with intratumoral vascular invasion than patients with 20 or less PY smoking history. The 5-year recurrence-free proportion for patients with greater than 20 PY smoking history was significantly lower than for patients with 20 or less PY smoking history (89.8% and 92.4%, respectively; $p = 0.033$).

Table 3. Pathological Characteristics and Outcomes

Characteristics	No. of Patients (%)	5-Year Overall Survival Rate (%)	Univariate *p* Value	Multivariate Analysis		
				HR	95% CI	*p* Value
Total	1,070	80.3				
Maximal dimension of resected tumor (cm)						
<2.0	518 (48)	86.7	<0.001[a]	1		
>2.0	552 (52)	74.3		1.2	0.911–1.581	0.195
Histologic differentiation						
Well-differentiated	456 (43)	91.5	<0.001[a]	1		
Moderately/poorly differentiated	614 (57)	70.9		1.573	1.114–2.22	0.01[a]
Lymphatic permeation						
Absent	837 (78)	88.1	<0.001[a]	1		
Present	233 (22)	56.4		1.548	1.15–2.083	0.004[a]
Intratumoral vascular invasion						
Absent	754 (70)	89.5	<0.001[a]	1		
Present	316 (30)	60		1.59	1.168–2.163	0.003*
Visceral pleural invasion						
Absent	841 (79)	87.2	<0.001[a]	1		
Present	229 (21)	56.2		1.684	1.272–2.230	<0.001[a]
N status						
N0	902 (84)	88.2	<0.001[a]	1		
N1–N3	168 (16)	41.2		3.247	2.395–4.401	<0.001[a]
Intrapulmonary metastasis						
Absent	1,037 (97)	80.9	0.001[a]	1		
Present	33 (3)	64.8		1.266	0.758–2.115	0.367

[a] Indicates significance.

CI = confidence interval; HR = hazard ratio for death.

Table 4. Correlation Between Smoking History and Pathological Characteristics in Entire Cohort

Characteristics	Smoking History: 0 ≤ PY ≤ 20	Smoking History: PY > 20	*p* Value[a]
Total	677	393	
	Number of patients (%)		
Maximal dimension of resected tumor (cm)			
<2.0	328 (48)	190 (48)	0.974
>2.0	349 (52)	203 (52)	
Histologic differentiation			
Well-differentiated	356 (53)	100 (25)	<0.001[b]
Moderately/poorly differentiated	321 (47)	293 (75)	
Lymphatic permeation			
Absent	545 (81)	292 (74)	0.018[b]
Present	132 (19)	101 (26)	
Intratumoral vascular invasion			
Absent	520 (77)	234 (60)	<0.001[b]
Present	157 (23)	159 (40)	
Pleural invasion			
Absent	545 (81)	296 (75)	0.046[b]
Present	132 (19)	97 (25)	
N status			
N0	593 (88)	309 (79)	<0.001[b]
N1–N3	84 (12)	84 (21)	
Intrapulmonary metastasis			
Absent	656 (97)	381 (97)	0.965
Present	21 (3)	12 (3)	

[a] χ^2 test. [b] Indicates significance, numbers in parentheses are percentages.

PY = pack-years.

Comment

Cigarette smoking is a well-known habitual risk factor in lung carcinogenesis [1]. Whether smoking-related lung cancer behaves more aggressively or results in poorer survival than lung cancer not related to smoking remains unclear however. To clarify the characteristics of smoking-related lung cancer, we assessed relationships between smoking history, pathological characteristics, and survival of patients with clinical stage IA lung adenocarcinoma.

Several studies have found that among patients with lung adenocarcinoma, smokers had significantly poorer outcomes than patients who never smoked [10, 11]. The present study also showed that a heavy history of smoking was an independent unfavorable prognostic factor in patients with clinical stage IA adenocarcinoma. Patients with greater than 20 PY smoking history had an increased likelihood of tumors with poor histologic differentiation; furthermore, they were significantly likely to have lymphatic permeation, intratumoral vascular invasion, pleural invasion, and lymph node metastases.

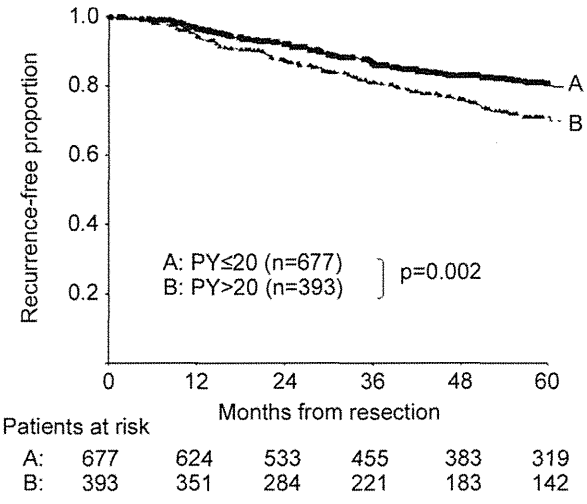

Fig 2. Recurrence-free proportion curves of patients with 20 or less pack-years (PY) smoking history and greater than 20 pack-years smoking history.

There may be several possible explanations for the highly aggressive and invasive biologic characteristics of pulmonary adenocarcinoma in patients with greater than 20 PY smoking history observed in this study. Cigarette smoke contains many mutagenic and carcinogenic chemicals that may be associated with mutations in tumor suppressor genes such as p53 [12–14]. Suzuki and colleagues [14] reported that tumors with p53 mutations exhibited an increased growth rate, which could explain why adenocarcinoma in patients with greater than 20 PY smoking history resulted in poorer outcomes compared with patients with 20 or less PY smoking history.

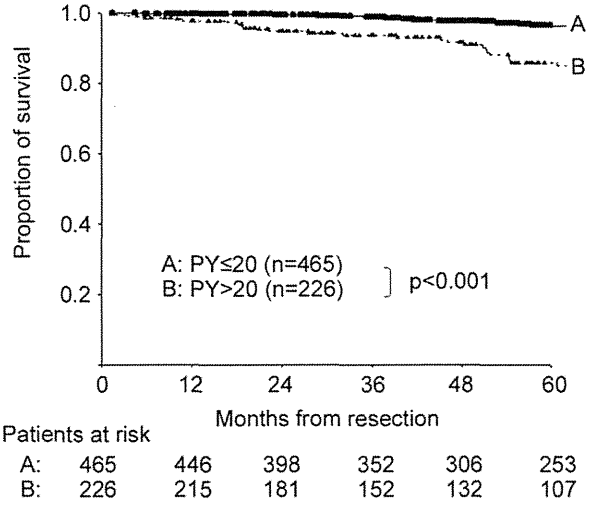

Fig 3. Overall survival curves of patients with 20 or less pack-years (PY) smoking history and greater than 20 pack-years smoking history in pathological stage IA patients.

Table 5. Correlation Between Smoking History and Pathological Characteristics in Pathological Stage IA Patients

Characteristics	Smoking History		
	0 ≤ PY ≤ 20	PY > 20	*p* Value[a]
Total	465	226	
	Number of patients (%)		
Maximal dimension of resected tumor (cm)			
<2.0	269 (58)	133 (59)	0.803
>2.0	196 (42)	93 (41)	
Histologic differentiation			
Well-differentiated	300 (65)	80 (35)	<0.001[b]
Moderately/poorly differentiated	165 (35)	146 (65)	
Lymphatic permeation			
Absent	428 (92)	196 (87)	0.061
Present	37 (8)	28 (13)	
Intratumoral vascular invasion			
Absent	416 (89)	173 (77)	<0.001[b]
Present	49 (11)	53 (23)	

[a] χ^2 test. [b] Indicates significance, numbers in parentheses are percentages.

PY = pack-years.

Several researchers recently reported successful results with limited surgical resection of clinical stage IA tumors [15–19]; however, locoregional recurrence is common even in patients with a pathologically confirmed negative surgical margin [20]. This recurrence could be attributed to intratumoral vessel involvement, when tumor cells spread into the surrounding parenchyma [21]. To minimize the risk of locoregional recurrence, appropriate selection criteria for limited resection should be established. These criteria can also dictate patient selection for standard lobectomy and node dissection, which could reduce the danger of missing nodal involvement and the opportunity to cure patients or administer adjuvant chemotherapy. In this study, heavy smoking was shown to be a significant predictor of unfavorable outcome that correlated with histologically invasive characteristics. Medical professionals should thus take care when proposing limited surgery to patients with both clinical stage IA adenocarcinoma and greater than 20 PY smoking history.

In the present study, 65% of patients with clinical stage IA lung adenocarcinoma were diagnosed with pathological stage IA. The 5-year survival rate of those patients with greater than 20 PY smoking history was significantly lower than patients with 20 or less PY smoking history. The 5-year recurrence-free proportion for patients with greater than 20 PY smoking history was also significantly lower than for patients with 20 or less PY smoking history. Patients with greater than 20 PY smoking history had a significantly high number of moderately or poorly differentiated carcinomas and carcinomas with intratumoral vascular invasions. Finally, patients with pathological stage IA tumors and greater than 20 PY smoking history had highly aggressive and invasive adenocarcinomas, which resulted in poorer outcomes than patients with 20 or less PY smoking history. A history of heavy smoking can thus be a simple clinical indicator for poor postoperative prognosis in patients with pathological stage IA adenocarcinoma because of its biologic aggressiveness and invasiveness.

This retrospective study had some limitations. One was a lack of diversity in the study population attributable to patient selection from a single Japanese institution and having only Japanese subjects. Another was that data on patient exposure to environmental tobacco smoke were not collected. Despite these limitations, our results clearly show that smoking history is highly associated with tumor invasiveness and poor outcomes in patients with clinical stage IA adenocarcinoma.

In conclusion, in the case of patients with clinical stage IA adenocarcinoma, a heavy history of smoking was associated with poor outcomes, making it a significant predictor of histologic tumor invasiveness.

The authors thank Prof J. Patrick Barron and Roderick J. Turner of the Department of International Medical Communications of Tokyo Medical University for their review of this manuscript. The work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan.

References

1. Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, Hainaut P. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. Oncogene 2002;21:7435–51.
2. Khuder SA. Effect of cigarette smoking on major histological types of lung cancer: a meta-analysis. Lung Cancer 2001;31:139–48.
3. Devesa SS, Bray F, Vizcaino AP, Parkin DM. International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. Int J Cancer 2005;117:294–9.
4. Sobue T, Yamamoto S, Hara M, Sasazuki S, Sasaki S, Tsugane S; JPHC Study Group, Japanese Public Health Center. Cigarette smoking and subsequent risk of lung cancer by histologic type in middle-aged Japanese men and women: the JPHC study. Int J Cancer 2002;99:245–51.
5. Yun YH, Lim MK, Jung KW, et al. Relative and absolute risks of cigarette smoking on major histologic types of lung cancer in Korean men. Cancer Epidemiol Biomarkers Prev 2005;14:2125–30.
6. Mansour Z, Kochetkova EA, Santelmo N, et al. Risk factors for early mortality and morbidity after pneumonectomy: a reappraisal. Ann Thorac Surg 2009;88:1737–43.
7. Licker MJ, Widikker I, Robert J, et al. Operative mortality and respiratory complications after lung resection for cancer: impact of chronic obstructive pulmonary disease and time trends. Ann Thorac Surg 2006;81:1830–7.
8. Travis WD, Brambilla E, Müller-Hermelink HK, Harris CC. World Health Organization classification of tumors: pathology and genetics of tumors of the lung, pleura, thymus and heart. IARC Press: Lyon; 2004.
9. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2007;2:706–14.
10. Nordquist LT, Simon GR, Cantor A, Alberts WM, Bepler G. Improved survival in never-smokers vs current smokers with primary adenocarcinoma of the lung. Chest 2004;126:347–51.

11. Gazdar AF, Minna JD. Cigarettes, sex, and lung adenocarcinoma. J Natl Cancer Inst 1997;89:1563–5.
12. Westra WH, Slebos RJ, Offerhaus GJ, et al. K-ras oncogene activation in lung adenocarcinomas from former smokers. Evidence that K-ras mutations are an early and irreversible event in the development of adenocarcinoma of the lung. Cancer 1993;72:432–8.
13. Suzuki H, Takahashi T, Kuroishi T, et al. p53 mutations in non-small cell lung cancer in Japan: association between mutations and smoking. Cancer Res 1992;52:734–6.
14. Ishida H, Irie K, Itoh T, Furukawa T, Tokunaga O. The prognostic significance of p53 and bcl-2 expression in lung adenocarcinoma and its correlation with Ki-67 growth fraction. Cancer 1997;80:1034–45.
15. Tsubota N, Ayabe K, Doi O, et al. Ongoing prospective study of segmentectomy for small lung tumors. Study Group of Extended Segmentectomy for Small Lung Tumor. Ann Thorac Surg 1998;66:1787–90.
16. Bando T, Miyahara R, Sakai H, et al. A follow-up report on a new method of segmental resection for small-sized early lung cancer. Lung Cancer 2009;63:58–62.
17. Yoshikawa K, Tsubota N, Kodama K, Ayabe H, Taki T, Mori T. Prospective study of extended segmentectomy for small lung tumors: the final report. Ann Thorac Surg 2002;73:1055–9.
18. Koike T, Yamato Y, Yoshiya K, Shimoyama T, Suzuki R. Intentional limited pulmonary resection for peripheral T1 N0 M0 small-sized lung cancer. J Thorac Cardiovasc Surg 2003;125:924–8.
19. Okada M, Koike T, Higashiyama M, Yamato Y, Kodama K, Tsubota N. Radical sublobar resection for small-sized non-small cell lung cancer: a multicenter study. J Thorac Cardiovasc Surg 2006;132:769–75.
20. Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. Lung Cancer Study Group. Ann Thorac Surg 1995;60:615–23.
21. Ichinose Y, Yano T, Yokoyama H, Inoue T, Asoh H, Katsuda Y. The correlation between tumor size and lymphatic vessel invasion in resected peripheral stage I non-small-cell lung cancer. A potential risk of limited resection. J Thorac Cardiovasc Surg 1994;108:684–6.

GENERAL THORACIC

INVITED COMMENTARY

It seems pretty clear now that heavy smoking not only causes lung cancer but also worsens the prognosis of patients with lung cancer when compared with light smokers or never-smokers. This reduction in survival for heavy smokers has been demonstrated not only in early-stage lung cancer [1, 2] but also in advanced-stage lung cancer [3]. In this retrospective review [4], the authors add to this body of literature by describing a cohort of more than 1,000 patients with resected clinical stage IA adenocarcinoma. They were able to show that a history of more than 20 pack-years of smoking was associated with worse survival, more poorly differentiated tumors, lymphatic permeation, vascular invasion, pleural invasion, and higher N status. The main findings of worse survival and worse histologic features held up even if only patients with pathologic stage IA disease were analyzed. The mechanisms responsible for these findings are not addressed in this study, but with further genomic analysis, they may be able to be elucidated.

An acknowledged limitation of this article for those of us practicing in the West is that all patients in the study were Japanese. Our lung cancer populations are somewhat different, as illustrated by the proportion of patients in this study who were never-smokers. Forty-nine percent of the total cohort (528/1070) and 78% of group that smoked less than or equal to 20 pack-years were never-smokers. Western early-stage lung cancer series typically have closer to 20% never-smokers. The group that smoked less than or equal to 20 pack-years is likely to be enriched with patients with an epidermal growth factor receptor mutation that may portend a better prognosis. Five-year survival of patients with pathologic stage IA ranged between 85.9% and 96.5% for heavy and light smokers, respectively. These are laudable survival statistics but ones rarely achieved in Western series.

What are the clinical implications of this study? It seems prudent to follow resected patients with stage IA disease and a heavy smoking history closely, as the biological characteristics of their tumors are worse. This may mean following patients after resected early-stage lung cancers more closely than the National Comprehensive Cancer Network recommends (ie, history and physical examination and chest computed tomography every 6–12 months for 2 years and then yearly thereafter). The authors speculate in the discussion section that a heavy smoking history may be a relative contraindication to a sublobar resection for lung cancer. Perhaps at the conclusion of CALGB 140503, *A Randomized Trial of Lobar vs Sublobar Resection for Small, Peripheral NSCLC*, a subset analysis can help answer this question. Additionally, it would be interesting to see if the inverse relationship between pack-years and survival hold true in a large multiinstitutional database and should underscore our efforts to get the Society of Thoracic Surgeons' General Thoracic Surgery Database linked with another database that can provide long-term survival data for our cancer patients.

William R. Burfeind, Jr, MD

Thoracic Surgery
St. Luke's Health Network
701 Ostrum St, Ste 201
Bethlehem, PA 18015
e-mail: burfeiw@slhn.org

References

1. Yoshino I, Dawano D, Oba T, et al. Smoking status as a prognostic factor in patients with stage I pulmonary adenocarcinoma. Ann Thorac Surg 2006;81:1189–93.
2. Bryant A, Cerfolio R. Differences in epidemiology, histology, and survival between cigarette smokers and never-smokers who develop non-small cell lung cancer. Chest 2007;132:185–92.
3. Janjigian Y, McDonnell K, Kris M, et al. Pack-years of cigarette smoking as a prognostic factor in patients with stage IIIB/IV non-small cell lung cancer. Cancer 2010;116:670–5.
4. Maeda R, Yoshida J, Ishii G, Hishida T, Nishimura M, Nagai K. Influence of cigarette smoking on survival and tumor invasiveness in clinical stage IA lung adenocarcinoma. Ann Thorac Surg 2012;93:1626–32.

0003-4975/$36.00
doi:10.1016/j.athoracsur.2012.02.034

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2012;42(3)189–195
doi:10.1093/jjco/hyr188
Advance Access Publication 30 December 2011

Differences Between Squamous Cell Carcinoma and Adenocarcinoma of the Lung: Are Adenocarcinoma and Squamous Cell Carcinoma Prognostically Equal?

Akikazu Kawase[1], Junji Yoshida[1,*], Genichiro Ishii[2], Masayuki Nakao[1], Keiju Aokage[1], Tomoyuki Hishida[1], Mitsuyo Nishimura[1] and Kanji Nagai[1]

[1]Division of Thoracic Surgery, National Cancer Center Hospital East and [2]Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan

*For reprints and all correspondence: Junji Yoshida, Division of Thoracic Surgery, National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa, Chiba 277-8577, Japan. E-mail: jyoshida@east.ncc.go.jp

Received August 8, 2011; accepted November 24, 2011

Objective: We analyzed pulmonary squamous cell carcinoma and adenocarcinoma patient survival in our single institution database, to evaluate the relationship of histologic analysis to survival and tumor aggressiveness.
Methods: We reviewed 1856 consecutive patients with surgically resected pulmonary squamous cell carcinoma or adenocarcinoma regarding their clinicopathologic characteristics, overall survival and recurrence-free proportion.
Results: In squamous cell carcinoma patients, there were more elderly male smokers and more patients with T2–4 tumors, moderately/poorly differentiated tumors, lymph node metastasis or vascular invasion than in adenocarcinoma patients. In all patients and in pN0 patients, patients with squamous cell carcinoma showed significantly poorer overall survival than those with adenocarcinoma, but there were no statistically significant differences in the recurrence-free proportion between the two histologic types. There were statistically significantly more lung cancer-specific deaths in patients with adenocarcinoma than in patients with squamous cell carcinoma ($P = 0.001$).
Conclusions: There were no differences in the development of recurrence between squamous cell carcinoma and adenocarcinoma of the lung, but considerable differences in overall survival were observed between the two histologic types. According to the stage grouping strategy of the TNM Classification for Lung and Pleural Tumours, these two histologic types need to be staged differently. This survival difference, however, may reflect the difference in patient background rather than in biologic aggressiveness between the two histologic types.

Key words: histologic type – prognosis – squamous cell carcinoma – adenocarcinoma – TNM classification

INTRODUCTION

Squamous cell carcinoma and adenocarcinoma are the two major histologic types of non-small cell lung cancer. Patients with adenocarcinoma were known to result in poorer prognosis than those with squamous cell carcinoma (1,2). However, a recent increase in the use of computed tomography (CT) has enabled small adenocarcinoma detection on a screening basis, and many of these small adenocarcinomas are relatively dormant bronchioloalveolar carcinomas and have favorable outcome (3). This may be one reason why patients with squamous cell carcinoma are known today to have a poorer prognosis than those with adenocarcinoma following surgical resection (4).

Squamous cell carcinoma mostly develops in smokers, in whom life-threatening co-morbidities often develop, which may also explain the poorer survival rates of patients with

squamous cell carcinoma compared with those with adenocarcinoma. However, differences in biological aggressiveness between squamous cell carcinoma and adenocarcinoma of the lung are not well understood.

In esophageal cancer staging, squamous cell carcinoma and adenocarcinoma are classified differently in the 7th Edition of the Cancer Staging Manual of the American Joint Committee on Cancer (5–7). In lung cancer, however, prognostic differences in histologic types are not taken into consideration in the latest TNM classification (8).

We retrospectively analyzed the survival differences between squamous cell carcinoma and adenocarcinoma of the lung, in an attempt to identify the prognostic impact of histologic difference and to incorporate it in future staging systems, based on our patient database.

PATIENTS AND METHODS

From July 1992 through December 2006, 1856 consecutive patients with pulmonary squamous cell carcinoma or adenocarcinoma underwent complete resection at our institution. We defined complete resection as segmentectomy or greater, with systematic ipsilateral hilar and mediastinal lymph node dissection but with no evidence of residual cancer either macroscopically or histologically. Patients who had induction chemotherapy, radiotherapy or both, patients with evidence of residual tumor at the surgical margin or patients with malignant effusion or distant metastasis verified intraoperatively or by means of postoperative pathologic examination were excluded from this study.

Cases were pathologically staged based on the 7th Edition of the TNM Classification for Lung and Pleural Tumours (8). Histopathologic studies were done according to the World Health Organization criteria (9). We reviewed the medical records of all patients for the following clinicopathologic factors: age, gender, smoking history (never or ever smoker), pathological differentiation, pathological T stage, pathological N stage, vascular invasion and lymphatic permeation.

Student's *t*-test was used to evaluate the relationships between histologic type (squamous cell carcinoma or adenocarcinoma) and age. Fisher's exact test was used to evaluate the relationships between histologic type and other clinicopathologic factors. We compared overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in all patients, in pN0 patients, in pT1N0 patients, in pT2N0 patients and in pT3/4N0 patients. When we analyzed recurrence-free proportion, we excluded 249 cases from this study because their recurrence data were incomplete. The survival rates and recurrence-free proportions were calculated using the Kaplan–Meier method, and univariate analyses were performed with the log-rank test. Multivariate analyses were performed by using the Cox proportional hazards model. Zero time was the date of pulmonary resection. The endpoint of overall survival was defined as the date of death from any cause, and the last follow-up observation was censored when the patient was alive or lost to follow-up. The endpoint of recurrence-free proportion was defined as the date when recurrence was confirmed. We examined patients at 3-month intervals for the first 2 years and at 6-month intervals thereafter on an outpatient basis. The follow-up evaluation included physical examination, chest radiography and blood examination including that of pertinent tumor markers. Further evaluations, including CT scans of the chest and abdomen, brain magnetic resonance imaging and bone scintigraphy, were performed on the detection of any symptoms or signs of recurrence. Since 2004, integrated positron emission tomography and CT have also been performed when appropriate. We diagnosed recurrence based on the findings of physical examination and diagnostic imaging and confirmed the diagnosis histologically when clinically feasible. The date of recurrence was defined as the date of cytohistological proof. However, in cases diagnosed on the basis of clinicoradiological findings, the date of recurrence was defined as the date of identification by a physician. The last follow-up observation was censored when the patient was recurrence-free or lost to follow-up. Patients who died from causes other than lung cancer recurrence were also censored on the date of death.

All P values were two-sided, and P values <0.05 were considered to represent statistically significant differences. Survival analyses were performed on SPSS software (Dr SPSS II for Windows, Standard Version 11.0, SPSS Inc., Chicago, IL, USA).

Data collection and analyses were approved, and the need to obtain written informed consent from each patient in this retrospective study was waived, by the institutional review board in June 2010.

RESULTS

Patient Characteristics

The patient characteristics are shown in Table 1. In squamous cell carcinoma patients, compared with adenocarcinoma patients, there were more elderly male smokers and more patients with T2–4 tumors, moderately/poorly differentiated tumors, lymph node metastasis or vascular invasion. In pN0 patients ($n = 1328$), there were more elderly male smokers and more patients with T2–4 tumors, moderately/poorly differentiated tumors or vascular invasion in squamous cell carcinoma patients.

Overall Survival Differences

Patients with squamous cell carcinoma showed significantly poorer overall survival than those with adenocarcinoma in all patients and in pN0 patients (Figs 1A and 2A). The results of multivariate analyses of the statistically significant characteristics listed in Table 1 are summarized in Table 2. Age, smoking history, pathological T classification, vascular

Table 1. Patient characteristics

Patient characteristics	All patients				pN0 patients			
	AD	SQ	*P*-value	Total	AD	SQ	*P*-value	Total
Age								
Median (range)	65 (32–90)	69 (31–88)	<0.001[a]		65 (32–90)	70 (31–88)	<0.001[a]	
Sex								
Men	731 (52)	418 (90)		1149 (62)	521 (51)	263 (89)		784 (59)
Women	662 (48)	45 (10)	<0.001[b]	707 (38)	510 (49)	34 (11)	<0.001[b]	544 (41)
Smoking history								
Never smoker	617 (44)	12 (3)		629 (34)	485 (47)	9 (3)		494 (37)
Ever smoker	776 (56)	451 (97)	<0.001[b]	1227 (66)	546 (53)	288 (97)	<0.001[b]	834 (63)
Pathological T classification								
T1a, T1b	689 (49)	131 (28)		820 (44)	602 (58)	103 (35)		705 (53)
T2a, T2b, T3, T4	704 (51)	332 (72)	<0.001[b]	1036 (56)	429 (42)	194 (65)	<0.001[b]	623 (47)
Pathological N classification								
N0	1031 (74)	297 (64)		1328 (72)	—	—	—	—
N1, N2	362 (26)	166 (36)	<0.001[b]	528 (28)	—	—	—	—
Pathological differentiation								
Well	491 (36)	21 (5)		512 (28)	454 (44)	17 (6)		471 (36)
Moderately/poorly	892 (74)	440 (95)	<0.001[b]	1332 (72)	569 (56)	279 (94)	<0.001[b]	848 (64)
Vascular invasion								
Absent	818 (59)	150 (32)		968 (52)	732 (71)	128 (43)		860 (65)
Present	575 (41)	313 (68)	<0.001[b]	888 (48)	299 (29)	169 (57)	<0.001[b]	468 (35)
Lymphatic permeation								
Absent	964 (69)	320 (69)		1284 (69)	847 (82)	238 (80)		1085 (82)
Present	429 (31)	143 (31)	1.000[b]	572 (31)	184 (18)	59 (20)	0.444[b]	243 (18)
Total	1393	463		1856	1031	297		1328

AD, adenocarcinoma; SQ, squamous cell carcinoma; T/N classification according to the 7th Edition of the TNM Classification for Lung and Pleural Tumours; numbers in parentheses are percentages.
[a]Student's t-test.
[b]Fisher's exact test.

invasion and lymphatic permeation were significant prognostic factors in all patients and in pN0 patients. Pathological N classification was a significant prognostic factor in all patients. Sex, pathological differentiation and histologic type were not significant prognostic factors in any patients or in pN0 patients.

Although patients with squamous cell carcinoma showed significantly poorer overall survival than those with adenocarcinoma in pT1N0 patients and in pT2N0 patients (Fig. 3A and C), no statistically significant differences were observed in pT3/4N0 patients ($P = 0.841$; Fig. 3E).

Recurrence-free Proportion Differences

There were no statistically significant differences in recurrence-free proportion between adenocarcinoma and squamous cell carcinoma in any patients ($P = 0.351$; Fig. 1B) or in pN0 patients ($P = 0.715$; Fig. 2B).

In pT1N0 patients, patients with squamous cell carcinoma showed significantly poorer recurrence-free proportion than those with adenocarcinoma (Fig. 3B). In pT2N0 patients, there was no statistically significant difference in recurrence-free proportion between the two histologic types ($P = 0.098$; Fig. 3D). In pT3/4N0 patients, patients with adenocarcinoma showed significantly poorer recurrence-free proportion than those with squamous cell carcinoma (Fig. 3F).

Causes of Death

There were 638 patients whose causes of death were identified in our cohort. There were significantly more lung cancer-specific deaths in adenocarcinoma patients than in squamous cell carcinoma patients ($P = 0.001$; Table 3).

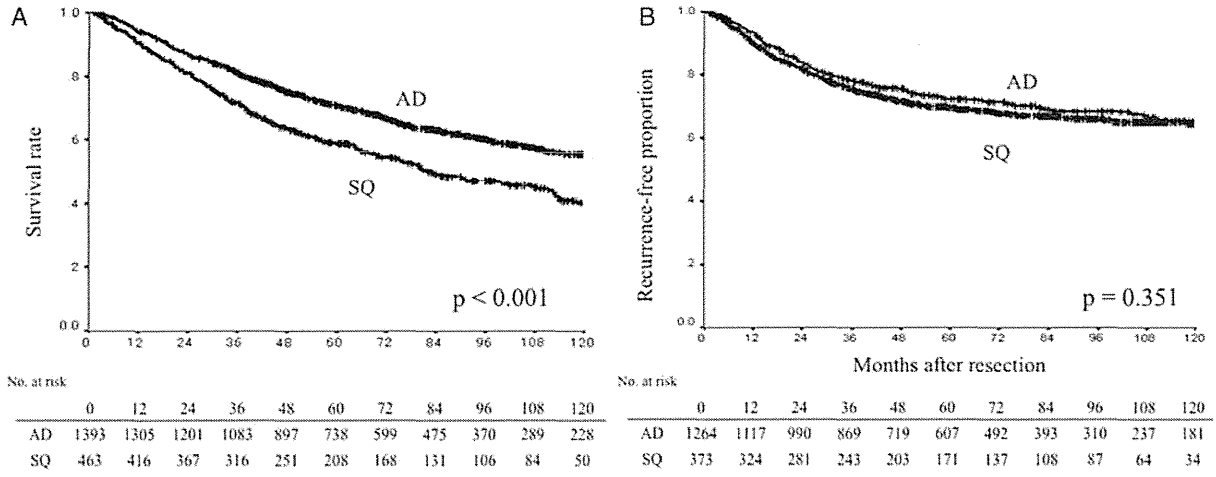

Figure 1. Overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in all patients. (A) Overall survival and (B) recurrence-free proportion curves of squamous cell carcinoma and adenocarcinoma in all patients. AD, adenocarcinoma; SQ, squamous cell carcinoma.

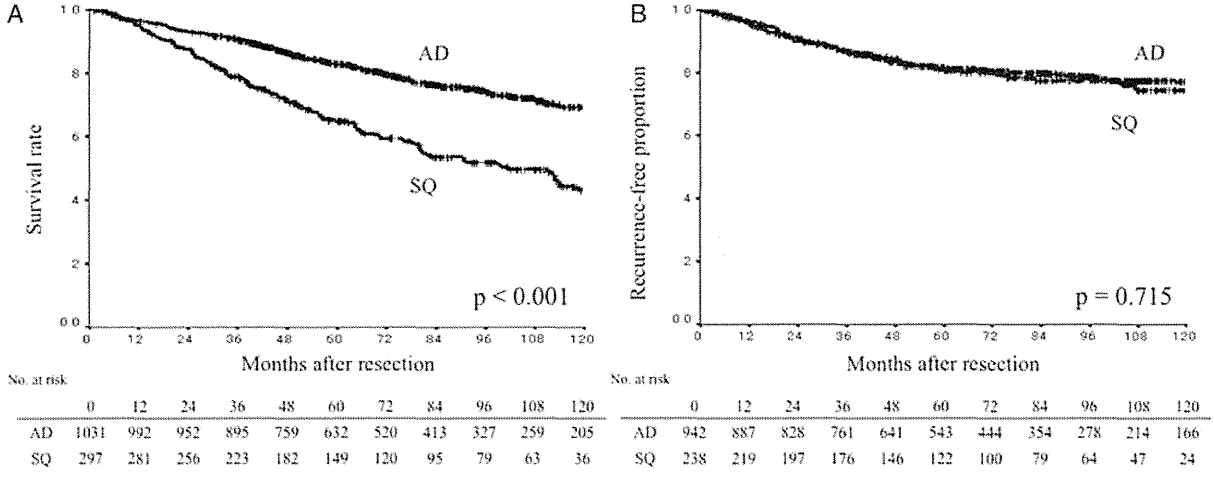

Figure 2. Overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in pN0 patients. (A) Overall survival and (B) recurrence-free proportion curves of squamous cell carcinoma and adenocarcinoma in pN0 patients.

Table 2. Multivariate analyses of overall survival

Patient characteristics	All patients		pN0	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (>65/≤65)	1.641 (1.414–1.905)	<0.001	2.152 (1.728–2.680)	<0.001
Sex (men/women)	1.023 (0.805–1.300)	0.853	1.054 (0.768–1.446)	0.744
Smoking history (ever smoker/never smoker)	1.429 (1.104–1.848)	0.007	1.661 (1.166–2.365)	0.005
Pathological T stage (T2 + 3 + 4/T1)	1.988 (1.653–2.391)	<0.001	2.267 (1.772–2.900)	<0.001
Pathological N stage (N1 + 2/N0)	2.182 (1.844–2.582)	<0.001	—	—
Pathological differentiation (moderately + poorly/well)	1.185 (0.943–1.490)	0.145	1.180 (0.888–1.567)	0.255
Vascular invasion (present/absent)	1.572 (1.301–1.900)	<0.001	1.811 (1.426–2.301)	<0.001
Lymphatic permeation (present/absent)	1.352 (1.148–1.592)	<0.001	1.375 (1.092–1.731)	0.007
Histologic type (SQ/AD)	0.875 (0.737–1.039)	0.128	1.095 (0.866–1.385)	0.448

HR, hazard ratio for death; CI, confidence interval.

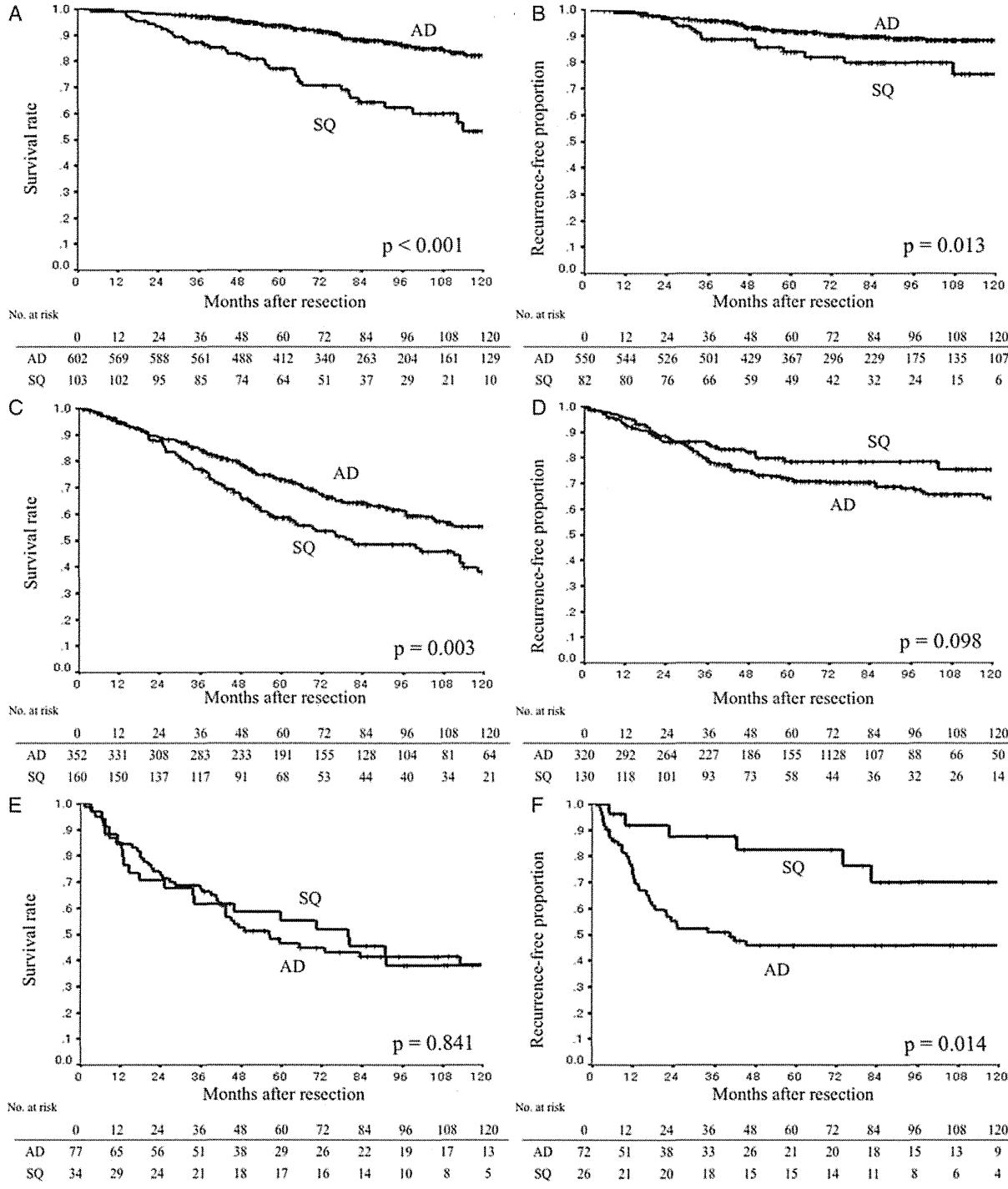

Figure 3. Overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in pT1N0 patients, in pT2N0 patients and in pT3/4N0 patients. (A) Overall survival and (B) recurrence-free proportion curves in pT1N0 patients. (C) Overall survival and (D) recurrence-free proportion curves in pT2N0 patients. (E) Overall survival and (F) recurrence-free proportion curves in pT3/4N0 patients.

DISCUSSION

We set out to determine the relationship of histologic analysis to survival and tumor aggressiveness in pulmonary squamous cell carcinoma and adenocarcinoma. Patients with pulmonary squamous cell carcinoma are known today to have a poorer prognosis than those with adenocarcinoma after surgical resection (4). Squamous cell carcinoma mostly develops in smokers

Table 3. Causes of death

Characteristics	Total	AD	SQ	*P* value
Lung cancer-specific deaths	479	355 (79)	124 (66)	
Deaths from other causes	159	96 (21)	63 (34)	0.001[a]
Total	638	451	187	

[a]Fisher's exact test; numbers in parentheses are percentages.

in whom life-threatening co-morbidities also often develop, including atherosclerotic cardiovascular events, chronic obstructive pulmonary disease and cerebral infarction (10), which may explain the poorer survival of patients with squamous cell carcinoma compared with those with adenocarcinoma. In the present study, there were significantly more patients who died of causes other than lung cancer in squamous cell carcinoma than in adenocarcinoma. However, it remains unclear whether biological aggressiveness differs between squamous cell carcinoma and adenocarcinoma of the lung.

In the present study, there were significantly more patients with squamous cell carcinoma than those with adenocarcinoma among smokers. In patients with squamous cell carcinoma, there were significantly more T2–4 patients and patients with lymph node metastases or vascular invasion. There were statistically significant differences in overall survival between adenocarcinoma and squamous cell carcinoma patients in all patients and in pN0 patient cohorts. However, when we analyzed recurrence-free proportion to exclude any possible influence of non-cancer-specific death and to compare biological aggressiveness between squamous cell carcinoma and adenocarcinoma, we found that there were no statistically significant differences in any patients or in pN0 patients. There were significantly more deaths from causes other than lung cancer in patients with squamous cell carcinoma than in those with adenocarcinoma.

These results indicate that although squamous cell carcinoma developed more frequently among smokers and was more advanced and invasive when resected compared with adenocarcinoma, its biological aggressiveness was not significantly different from adenocarcinoma. The poorer overall survival in patients with squamous cell carcinoma than those with adenocarcinoma seemed to be attributable to advanced and invasive cancer status on resection and smoking/age-related co-morbidities.

We also analyzed overall survival and recurrence-free proportion in each pathological T stage in pN0 patients to compare biological aggressiveness between squamous cell carcinoma and adenocarcinoma in each T stage. In pT1N0 patients, the patients with squamous cell carcinoma had significantly poorer survival and recurrence-free proportion than patients with adenocarcinoma. This may partly be explained by the fact that a considerable number of pT1 adenocarcinoma patients had non- or minimally invasive disease, such as bronchioloalveolar carcinoma, thereby resulting in better outcome compared with squamous cell carcinoma patients. In pT3/4 patients, on the other hand, there was no significant difference in overall survival between the two histologic types, but adenocarcinoma patients had significantly poorer recurrence-free proportion than squamous cell carcinoma patients. The poorer recurrence-free proportion of adenocarcinoma patients compared with squamous cell carcinoma patients may be interpreted that adenocarcinoma of this T status has biologically more aggressive nature than squamous cell carcinoma. However, probably because squamous cell carcinoma patients had more smoking/age-related co-morbidities and were more often killed by them than adenocarcinoma patients, there was no significant difference in overall survival.

In the 7th Edition of the TNM Classification for Lung and Pleural Tumours, stage groupings are based on overall survival (8). According to the strategy in this study, and based on our findings, squamous cell carcinoma and adenocarcinoma of the lung need to be staged differently. It is important to note, however, that the difference is likely to be due to advanced and invasive cancer status on resection and smoking/age-related co-morbidities of patients with squamous cell carcinoma, but not to biological tumor aggressiveness of squamous cell carcinoma.

There were several limitations in this study. Although the total number of consecutive patients was large (1856), the study was performed in a single institution using a homogenous Japanese ethnic group. Therefore, a multicenter trial based on various ethnic groups may be valuable. There were more well-differentiated tumors in adenocarcinomas than in squamous cell carcinomas in the present cohort. This may be another reason for the observed better prognosis in adenocarcinoma patients than in squamous cell carcinoma patients.

In conclusion, this study showed that there were no differences in the development of recurrence between squamous cell carcinoma and adenocarcinoma of the lung, but considerable differences in overall survival were observed between the two histologic types in all patients and pN0 patients. According to the stage grouping strategy of the TNM Classification for Lung and Pleural Tumours, these two histologic types need to be staged differently. This survival difference, however, may reflect the difference in patient background rather than the difference in biological aggressiveness between the two histologic types.

Acknowledgements

The authors thank Mr Roderick J. Turner and Prof. J. Patrick Barron of the Department of International Medical Communications of Tokyo Medical University for their review of this manuscript.

Funding

The work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan.

Conflict of interest statement

None declared.

References

1. Ichinose Y, Yano T, Asoh H, Yokoyama H, Yoshino I, Katsuda Y. Prognostic factors obtained by a pathologic examination in completely resected non-small-cell lung cancer. An analysis in each pathologic stage. *J Thorac Cardiovasc Surg* 1995;110:601–5.
2. Suzuki K, Nagai K, Yoshida J, Nishimura M, Takahashi K, Yokose T, et al. Conventional clinicopathologic prognostic factors in surgically resected nonsmall cell lung carcinoma. A comparison of prognostic factors for each pathologic TNM stage based on multivariate analyses. *Cancer* 1999;86:1976–84.
3. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995;75:2844–52.
4. Asamura H, Goya T, Koshiishi Y, Sohara Y, Eguchi K, Mori M, et al. A Japanese Lung Cancer Registry study: prognosis of 13,010 resected lung cancers. *J Thorac Oncol* 2008;3:46–52.
5. Rice TW, Blackstone EH, Rusch VW. A cancer staging primer: esophagus and esophagogastric junction. *J Thorac Cardiovasc Surg* 2010;139:527–9.
6. Rice TW, Blackstone EH, Rusch VW. 7th edition of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction. *Ann Surg Oncol* 2010;17:1721–4.
7. Rice TW, Rusch VW, Apperson-Hansen C, Allen MS, Chen LQ, Hunter JG, et al. Worldwide esophageal cancer collaboration. *Dis Esophagus* 2009;22:1–8.
8. Goldstraw P, editor. *Staging Manual in Thoracic Oncology*. Denver: IASLC 2009.
9. Travis WD, Brambilla E, Müller-Hermelink H, Harris C, editors. *Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press 2004.
10. Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. *BMJ* 2004;328:1519.

Number of Circulating Endothelial Progenitor Cells and Intratumoral Microvessel Density in Non-small Cell Lung Cancer Patients

Differences in Angiogenic Status between Adenocarcinoma Histologic Subtypes

Ryo Maeda, MD,†‡ Genichiro Ishii, MD,* Masami Ito, MD,*‡ Tomoyuki Hishida, MD,‡ Junji Yoshida, MD,‡ Mitsuyo Nishimura, MD,‡ Hironori Haga, MD,† Kanji Nagai, MD,‡ and Atsushi Ochiai, MD**

Introduction: Angiogenesis plays a significant role in tumor progression. This study examined the association between the number of circulating endothelial progenitor cells (EPCs), intratumoral microvessel density (MVD) (both of which may be markers for neovascularization), and lung cancer histological types, particularly adenocarcinoma histological subtypes.

Methods: A total of 83 stage I non-small cell lung cancer (NSCLC) patients underwent complete tumor resection between November 2009 and July 2010. The number of EPCs from the pulmonary artery of the resected lungs was measured by assaying $CD34^+$/vascular endothelial growth factor receptor 2 positive cells, and the MVD was assessed immunohistochemically in tumor specimens by staining for CD34.

Results: A statistically significant correlation between the number of EPCs from pulmonary artery and intratumoral MVD was found ($p < 0.001$). No statistically significant differences in the number of EPCs and the MVD were observed between the adenocarcinomas and the squamous cell carcinomas. Among the adenocarcinoma histological subtypes, a higher number of EPCs and MVD were found significantly more frequently in solid adenocarcinomas than in nonsolid adenocarcinomas ($p < 0.001$ and $p = 0.011$, respectively). In addition, solid adenocarcinomas showed higher levels of vascular endothelial growth factor using quantitative real-time polymerase chain reaction in the tumor tissue samples than in the nonsolid adenocarcinomas ($p = 0.005$).

Conclusion: The higher number of circulating EPCs and the MVD of solid adenocarcinoma may indicate the presence of differences in the tumor angiogenic status between early-stage adenocarcinoma histological subtypes. Among adenocarcinoma patients, patients with solid adenocarcinoma may be the best candidates for antiangiogenic therapies.

Key Words: Non-small cell lung cancer, Adenocarcinoma, Subtypes, Angiogenesis, Circulating endothelial progenitor cell.

(*J Thorac Oncol.* 2012;7: 503–511)

Lung cancer is the leading cause of death from cancer in the world,[1] and non-small cell lung cancer (NSCLC) accounts for the majority of lung cancers.[2] Among NSCLC histological types, adenocarcinoma of the lung is the most frequent histological type, and its incidence is increasing in most countries.[3] In Japan, adenocarcinoma is the most common histological type of resected lung cancer, accounting for more than 60% of all cases.[4]

Adenocarcinomas are categorized into four histological subtypes based on morphology: bronchioloalveolar carcinoma (BAC), acinar, papillary, and solid adenocarcinoma.[5] Among these major histological subtypes of lung adenocarcinoma, BAC is often reported to be associated with a favorable prognosis,[6–8] whereas the other subtypes are considered invasive and are associated with unfavorable outcomes.[9,10]

Treatment of lung adenocarcinoma is now moving beyond conventional chemotherapy, with the advent of molecular-targeted therapies such as epidermal growth factor receptor (EGFR) inhibitors.[11] An EGFR mutation was found specifically in the BAC subtype, and differences in the frequency of EGFR mutations exist among the adenocarcinoma histological subtypes.[11]

Angiogenesis is essential for cancer growth and progression.[12] Another key feature of molecular-targeted therapies against lung adenocarcinoma is the inhibition of specific

*Department of Pathology, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan; †Department of Diagnostic Pathology, Kyoto University Hospital, Sakyo-ku, Kyoto, Japan; and ‡Department of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Genichiro Ishii, MD, PhD, or Atsushi Ochiai, MD, PhD, Pathology Division, Research Center for Innovative Oncology, National Cancer, Center Hospital East, Kashiwa, Chiba 277-8577, Japan. E-mail: gishii@east.ncc.go.jp or aochiai@east.ncc.go.jp

ISSN: 1556-0864/12/0703-0503

cytokines essential for tumor vascularization.[13] Although bevacizumab (Avastin; Genentech, South San Francisco, CA), a monoclonal antibody with an antiangiogenic effect that specifically antagonizes and blocks vascular endothelial growth factor (VEGF), had been shown to increase the overall survival of patients with advanced adenocarcinoma when administered in combination with standard chemotherapy,[13] which adenocarcinoma subtypes are most dependent on angiogenesis for their growth remains unclear.

Recent evidence shows that the tumor vasculature can arise through vasculogenesis, a process by which bone marrow–derived $CD34^+$/vascular endothelial growth factor receptor 2 $(VEGFR\text{-}2)^+$ endothelial progenitor cells (EPCs) are recruited and differentiated in situ into mature endothelial cells to form new blood vessels.[14,15] The level of circulating EPCs has been reported to be a potential marker for neovascularization and for the response to antiangiogenic therapies.[16] Other studies have also reported a significant correlation between neovascularization, as assessed by the intratumoral microvessel density (MVD), and patient outcome in a variety of tumors.[17–19]

The addition of antiangiogenic therapy to conventional chemotherapy has recently become a standard therapy for lung adenocarcinoma.[13] However, the exact relationship between specific adenocarcinoma subtypes and the number of EPCs or MVD, both of which may be markers for neovascularization or the response to antiangiogenic therapies, has not been addressed thus far. The evaluation of these relationships could lead to the development of a relatively simple pathological examination of lung adenocarcinoma to determine a patient's sensitivity to antiangiogenic-targeted treatment and may help to identify the biological differences between adenocarcinoma histological subtypes. This study investigated the association between the number of circulating EPCs, MVD, and lung cancer histological types, with particular emphasis on adenocarcinoma subtypes.

PATIENTS AND METHODS

Patients

A total of 122 consecutive Japanese patients with stage I NSCLC underwent complete tumor resection with a lobectomy or a more extensive surgery between November 2009 and July 2010 at the National Cancer Center Hospital East. Among these patients, 39 were excluded because of (1) preoperative chemotherapy and/or radiation therapy ($n = 2$) or (2) unavailability of blood samples from the resected lungs ($n = 37$). The remaining 83 patients were included in this study.

Pathological Evaluation

All surgical specimens were fixed with 10% formalin and embedded in paraffin wax. The tumors were cut at approximately 5-mm intervals, and serial 4-μm sections were stained with hematoxylin-eosin. The disease stages were diagnosed based on the tumor, node, and metastasis (TNM) classification of the International Union Against Cancer, 7th edition.[20] The histological type was determined according to the classification of the World Health Organization.[5] We diagnosed squamous cell carcinoma based on the findings of keratinization, intercellular bridges, and squamous pearl formation. Adenocarcinoma histological subtypes were categorized into BAC (nonmucinous or mucinous), papillary, acinar, and solid adenocarcinomas according to the classification of the World Health Organization.[5] Mucin production in a solid adenocarcinoma component was confirmed using the Alcian blue-periodic acid Schiff method. All the adenocarcinomas were divided according to the predominant subtype into four subgroups: BAC, papillary, acinar, and solid adenocarcinoma with mucin production. Tumor size was measured as the maximal diameter of the tumor on the cut sections. Intratumoral vascular invasion and visceral pleural invasion were evaluated using staining with hematoxylin-eosin and Victoria blue-van Gieson stains.

Blood Sampling from the Pulmonary Artery of Resected Specimens

Human monocytes were isolated from the pulmonary artery (PA) of resected lungs as previously reported.[21,22] In brief, the dissected and ligated PA of surgically resected lungs contains more than 4 ml of blood. In this study, a 21-gauge needle was inserted into the PA of 83 lungs surgically resected from stage I primary NSCLC patients at our hospital. All the specimens were collected after the patients had given their written informed consent, and the study was approved by the Institutional Review Board of the National Cancer Center.

Flow Cytometry

Blood samples from the PA of the resected lungs were processed within 1 hour after collection. Blood mononuclear cells from the PA were prepared by gradient centrifugation using Ficoll-Hypaque. The expression of cell surface antigens was determined using two-color immunofluorescence staining. In brief, 100 μl of blood mononuclear cells (containing 5×10^5 cells) were incubated with 40 μl of FcR-blocking reagent (MBL, Aichi, Japan) for 10 minutes to inhibit nonspecific bindings. Subsequently, the cells were incubated at 4°C for 15 minutes with 10 μl of phycoerythrin-conjugated antihuman CD34 mAb (BioLegend, Bergisch-Gladbach, Germany) and 20 μl of allophycocyanin-conjugated VEGFR-2 mAb (R&D Systems, Wiesbaden-Nordenstadt, Germany). Phycoerythrin (PE)- and allophycocyanin (APC)-conjugated isotype-matched immunoglobulin Ig-G1 (Abcam, Cambridge, United Kingdom) and Ig-G2a (DakoCytomation, Hamburg, Germany) antibodies were used as negative controls. The cells were washed three times to remove unbound antibodies and finally resuspended in 500 μl of fluorescence activated cell sorting (FACS) solution. A FACS analysis was performed using a FACSCalibur flow cytometer (BD Bioscience, Heidelberg, Germany). A minimum of 10,000 events were collected.

Immunohistochemistry

After reviewing the hematoxylin-eosin-stained slides of the surgical specimens, the block containing the most extensive tumor component was selected from each specimen. Sections (4 μm each) were cut from the paraffin blocks and

mounted on silanized slides. The sections were deparaffinized in xylene, dehydrated in a graded ethanol series, washed with distilled water, and placed in 0.1 M citric acid buffer. For antigen retrieval, the slides were heated at 95°C for 20 minutes in a microwave oven and then allowed to cool for 1 hour at room temperature. Next, the slides were washed three times in phosphate-buffered saline (PBS) and immersed in a 0.3% hydrogen peroxide solution in methanol for 15 minutes to inhibit endogenous peroxidase activity. After washing the slides three times in PBS, nonspecific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 30 minutes at room temperature. Individual slides were then incubated overnight at 4°C with mouse anti-CD34 antibody (R&D Systems) at a final dilution of 1:50 in blocking buffer. The slides were again washed three times with PBS, incubated with EnVision (DAKO, Tokyo, Japan) for 1 hour at room temperature, and after extensive washing with PBS, the color reaction was developed for 2 minutes in 2% 3,3′-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxide. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated, and mounted. The three most vascular areas (hot spots) in the invasive foci within a section were selected for the quantification of angiogenesis, and vessels labeled with the anti-CD34 mAb were counted under light microscopy at a magnification of ×400 (×400; ×40 objective, and ×10 ocular; 0.196 mm^2/field) based on previous reports.[18–23] Each single or connected endothelial cell that stained in these areas was counted as a microvessel. The average counts were recorded as the CD34-MVD for each case.

Tissue Samples, RNA Extraction, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA was extracted from 27 adenocarcinoma patients who had undergone surgical resection at our hospital. Samples of cancer tissue were collected and immediately homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) with Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and stored at −80°C until use. Total RNA was isolated from the tissues using a commercial RNA isolation reagent according to the manufacturer's instructions. The RNA was reverse transcribed to synthesize cDNA using a PrimerScript RT reagent kit according to the manufacturer's instructions (Takara Biochemicals, Shiga, Japan).

To quantitatively compare the mRNA level of VEGF-A, RT-PCR was performed in a Smart Cycler System (TaKaTa) using SYBR Premix Ex Taq (TaKaRa). The sense and antisense primers used for the quantitative amplification of VEGF mRNAs were 5′-GAGCCTTGCCTTGCTGCTC-TAC-3′ and 5′-CACCAGGGTCTCGATTGGATG-3′, and the primers used for the amplification of glyceraldehyde-3-phosphate dehydrogenase as an internal control were 5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-ATGGTGGT-GAAGACGCCAGT-3′.

The amount of template cDNA was expressed by a threshold cycle (G) that was determined from the amplification curve (exponential curve) and a threshold level of PCR product detection. One G was equal to a twofold difference in the initial template. The quantification data were analyzed using Smart Cycler System software, version 2.0d (Cepheid). The level of VEGF expression was reported as the ratio of its expression to the level of *GAPDH* gene expression in the same sample.

Clinicopathological Data

The medical records of all the patients were reviewed to obtain the clinicopathological data, which included age (dichotomized according to a median age of 69 years), sex, smoking history (nonsmokers or ever-smokers), diameter of the tumor on the resected specimens (dichotomized according to a diameter of 3.0 cm), histological type (adenocarcinoma, squamous cell carcinoma, or others), lymphatic permeation (present or absent), intratumoral vascular invasion (present or absent), and visceral pleural invasion (as defined in the TNM classification, 7th edition[17]; present or absent).

Data and Statistical Analysis

All data are presented as the mean ± SE. Differences in categorical outcomes were evaluated using the χ^2 test. Continuous variables were compared using t tests. All the reported p values were two-sided, and the significance level

TABLE 1. Patient Characteristics

Characteristics	No. of Patients (%)
Total	83 (100)
Age (yr)	
<69	41 (49)
≥69	42 (51)
Sex	
Women	30 (36)
Men	53 (64)
Smoking history	
Never-smoker	32 (39)
Ever-smoker	51 (61)
Tumor size (cm)	
≤3	62 (75)
>3	21 (25)
Histologic type	
Adenocarcinoma	63 (76)
Predominant subtype	
BAC	23
Papillary	12
Acinar	18
Solid	10
Squamous cell carcinoma	15 (18)
Others	5 (6)
Lymphatic permeation	
Absent	77 (93)
Present	6 (7)
Intratumoral vascular invasion	
Absent	59 (71)
Present	24 (29)
Visceral pleural invasion	
Absent	69 (83)
Present	14 (17)

BAC, bronchioloalveolar carcinoma.

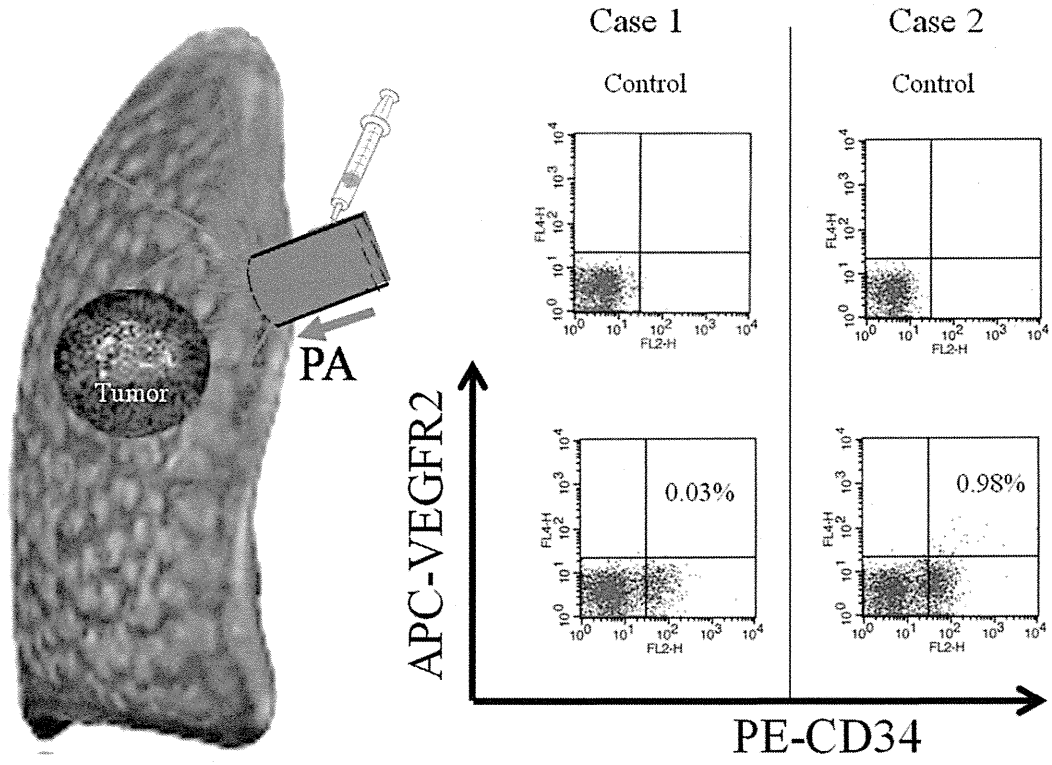

FIGURE 1. Quantification of circulating endothelial progenitor cells (EPCs) in blood mononuclear cells from the pulmonary fraction. Representative data from two flow cytometry analyses are shown. PA, pulmonary artery; VEGFR2, vascular endothelial growth factor receptor 2; APC, allophycocyanin; PE, phycoerythrin.

was set at less than 0.05. Analyses were performed using SPSS version 11.0 (Dr. SPSS II for Windows, standard version 11.0; SPSS Inc., Chicago, IL).

RESULTS

Patient Characteristics

This study analyzed 83 patients with completely resected stage I NSCLC, including 53 men and 30 women with a median (range) age of 69 years (42–82 years). Thirty-two patients were never-smokers, and 51 were ever-smokers. The patient characteristics are shown in Table 1.

Sixty-three patients had adenocarcinoma, 15 had squamous cell carcinoma, and 5 had other histological subtypes. The 63 patients with adenocarcinoma were classified according to the predominant subtypes as follows: 23 patients had BAC, and 12 patients had papillary, 18 patients had acinar, and 10 patients had solid adenocarcinomas. Of the 83 patients included in this study, 21, 6, 24, and 14 patients had tumors with a large tumor size (≥3 cm), lymphatic permeation, intratumoral vascular invasion, and pleural invasion, respectively (Table 1).

EPCs from PA in Stage I NSCLC and Correlation between Clinicopathological Characteristics and the Number of EPCs

Figure 1 shows the EPC quantification in blood mononuclear cells from the PA fraction and representative data for two flow cytometry analyses. Table 2 shows the number of EPCs from the PA fraction according to the clinicopathological features in all the patients. No statistically significant correlations were observed between the number of EPCs and the patient's age, sex, smoking history, tumor size, histological types, lymphatic permeation, vascular invasion, or visceral pleural invasion (Table 2).

MVD in Stage I NSCLC and Correlation between Clinicopathological Characteristics and MVD

The median microvessel count for all patients was 16.2 (per 400× field), with an interquartile range of 0 to 78.7. Representative tissue specimens from stage I NSCLC patients are shown in Figure 2 for adenocarcinomas with a low MVD (Figure *2A*) and a high MVD (Figure *2B*) and squamous cell carcinomas with a low MVD (Figure *2C*) and a high MVD (Figure *2D*). Patients were classified into two groups with a high MVD (>16.2) or a low MVD (≤16.2) based on the median MVD of the entire group.

Table 3 lists the MVD in correlation with the clinicopathological features in all the patients. No statistically significant correlations were observed between the MVD and the patient's age, sex, smoking history, tumor size, histological type, lymphatic permeation, vascular invasion, or visceral pleural invasion. In contrast, a statistically significant corre-

TABLE 2. Correlation between Clinicopathological Characteristics and the Number of EPCs in the Entire Cohort

Characteristics	No. of Patients (%)	No. of EPC from PA (/ml) ± SE	p
Total	83 (100)	2619 ± 372	
Age (yr)			
<69	41 (49)	2829 ± 569	0.580
≥69	42 (51)	2414 ± 486	
Sex			
Women	30 (36)	1730 ± 315	0.072
Men	53 (64)	3122 ± 546	
Smoking history			
Never-smoker	32 (39)	1768 ± 342	0.069
Ever-smoker	51 (61)	3153 ± 556	
Tumor size (cm)			
≤3	62 (75)	2456 ± 409	0.454
>3	21 (25)	3100 ± 846	
Histologic type			
Adenocarcinoma	63 (76)	2420 ± 397	
Squamous cell carcinoma	15 (18)	3527 ± 1204	0.272[a]
Others	5 (6)	2403 ± 429	0.991[a]
Lymphatic permeation			
Absent	77 (93)	2459 ± 358	0.123
Present	6 (7)	4678 ± 2345	
Intratumoral vascular invasion			
Absent	59 (71)	2193 ± 356	0.072
Present	24 (29)	3667 ± 923	
Visceral pleural invasion			
Absent	69 (83)	2480 ± 389	0.409
Present	14 (17)	3306 ± 1105	

[a] Compared with adenocarcinoma.
EPC, circulating endothelial progenitor cell; PA, pulmonary artery.

lation was observed only between the MVD and the number of EPCs ($p < 0.001$). In the high MVD group, the number of EPCs was significantly higher than that in the low MVD group (mean = 4380 ± 620 and 901 ± 185, respectively; Figure 3).

Correlation between the Number of EPCs and Adenocarcinoma Histological Subtypes

Figure 4*A* shows the number of EPCs in stage I adenocarcinoma patients stratified according to their predominant histological subtypes. The number of EPCs in patients with predominantly solid adenocarcinomas (mean = 5776 ± 1720/ml) was significantly higher than that among patients with predominantly BAC (mean = 1643 ± 420/ml, $p = 0.003$), papillary adenocarcinoma (mean = 2140 ± 770/ml, $p = 0.048$), or acinar adenocarcinoma (mean = 1734 ± 394/ml, $p = 0.007$).

No statistically significant differences in the number of EPCs were observed among patients with predominantly BAC, papillary adenocarcinoma, and acinar adenocarcinoma. Therefore, patients with predominantly BAC, papillary adenocarcinoma, or acinar adenocarcinoma were grouped as nonsolid adenocarcinomas patients and were compared with patients with predominantly solid adenocarcinomas in the following analyses (Figure *4B*).

Differences in MVD and VEGF Levels in Tumor Tissue Samples between Solid and Nonsolid Adenocarcinoma Patients as Determined Using Quantitative Real-Time PCR

Table 4 shows the differences in the MVD between solid and nonsolid adenocarcinoma patients. The high-MVD group included a significantly higher number of solid adenocarcinoma patients than those with nonsolid adenocarcinoma patients ($p = 0.011$).

Because VEGF-A has been reported to play a crucial role in the recruitment of EPCs[24,25] and angiogenesis can be assessed according to the MVD,[26,27] the level of mVEGF-A

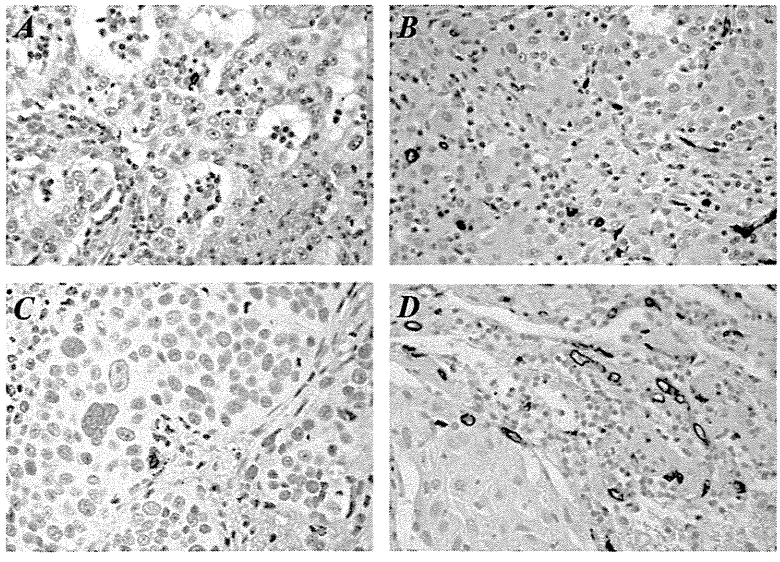

FIGURE 2. Immunohistochemical staining of non-small cell lung cancer (NSCLC) tissue with anti-CD34 antibodies for the quantification of microvessel density (MVD). Sections from specimens of adenocarcinoma with a low MVD (*A*) and a high MVD (*B*) and squamous cell carcinoma with a low MVD (*C*) and a high MVD (*D*) are shown. Original magnification, ×400. EPC, endothelial progenitor cell; PA, pulmonary artery.

TABLE 3. Correlation between Clinicopathological Characteristics and the Number of EPCs and MVD in the Entire Cohort

Characteristics	No. of Patients (%)	MVD		p
		Low[a]	High[b]	
Total	83 (100)	42	41	
Age (yr)				
<69	41 (49)	18 (44)	23 (56)	0.228
≥69	42 (51)	24 (57)	18 (43)	
Sex				
Women	30 (36)	17 (57)	13 (43)	0.406
Men	53 (64)	25 (47)	28 (53)	
Smoking history				
Never-smoker	32 (39)	19 (59)	13 (41)	0.205
Ever-smoker	51 (61)	23 (45)	28 (55)	
Tumor size (cm)				
≤3	62 (75)	32 (52)	30 (48)	0.752
>3	21 (25)	10 (48)	11 (52)	
Histologic type				
Adenocarcinoma	63 (76)	36 (57)	27 (43)	0.093
Squamous cell carcinoma	15 (18)	5 (33)	10 (67)	
Others	5 (6)	1 (20)	4 (80)	
Lymphatic permeation				
Absent	77 (93)	40 (52)	37 (48)	0.38
Present	6 (7)	2 (33)	4 (67)	
Intratumoral vascular invasion				
Absent	59 (71)	33 (56)	26 (44)	0.128
Present	24 (29)	9 (38)	15 (62)	
Visceral pleural invasion				
Absent	69 (83)	37 (54)	32 (46)	0.222
Present	14 (17)	5 (36)	9 (64)	
No. of EPC from PA/ml ± SE	83 (100)	901 ± 185	4380 ± 620	<0.001[c]

Numbers in parentheses are in percentages.
[a] ≤median.
[b] >median.
[c] Significance.
MVD, microvessel density; EPC, circulating endothelial progenitor cell; PA, pulmonary artery.

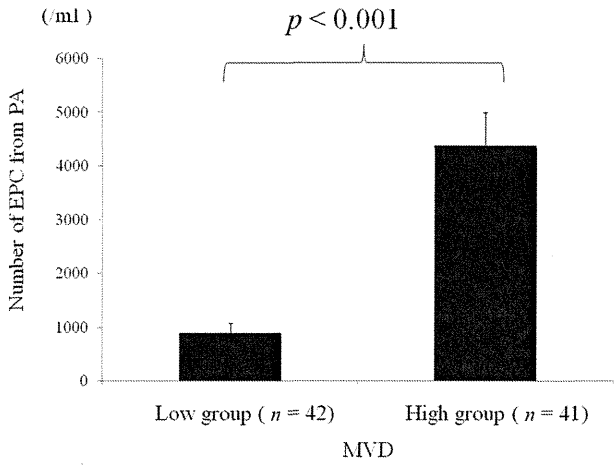

FIGURE 3. Correlation between the microvessel density (MVD) and the number of endothelial progenitor cells (EPCs) in the entire cohort. PA, pulmonary artery.

was measured using quantitative real-time PCR in the tumor tissue samples and was compared between solid and nonsolid adenocarcinoma patients. The level of mVEGF-A was significantly higher among solid adenocarcinomas than among nonsolid adenocarcinomas (Figure 5, $p = 0.005$).

DISCUSSION

In the most recently reported series, adenocarcinoma was found to be the most common type of early-stage lung cancer.[3,4] The recent increase in the detection of early-stage adenocarcinoma in Japan can be attributed to a nationwide mass screening system.[28] The major histological subtypes of adenocarcinoma are characterized as BAC, acinar, papillary, and solid components. Several reports have described differences in survival between adenocarcinoma subtypes. BAC has specific radiological features and is reported to be significantly correlated with a favorable prognosis.[6–8] In contrast, patients with solid adenocarcinoma have significantly poorer outcomes than those with other histological subtypes, and solid adenocarcinoma is

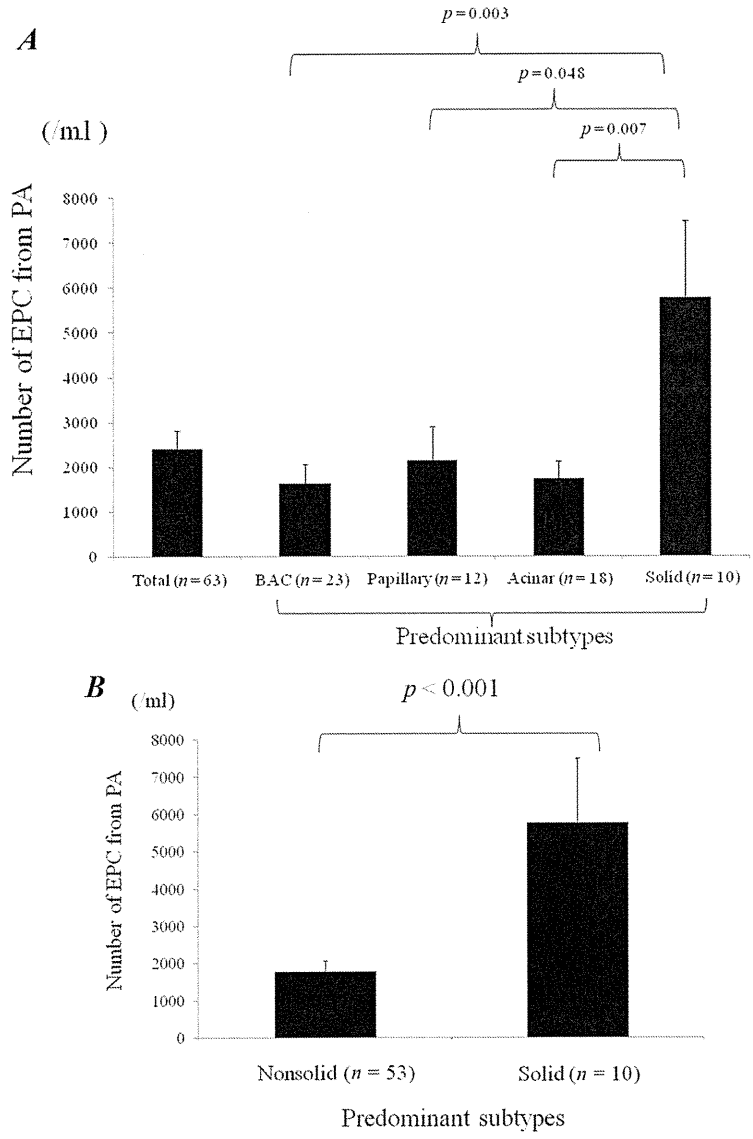

FIGURE 4. *A*, Correlation between the number of endothelial progenitor cells (EPCs) and adenocarcinoma histological subtypes. *B*, Differences in number of endothelial progenitor cells (EPCs) between solid and nonsolid adenocarcinoma patients. PA, pulmonary artery; BAC, bronchioloalveolar carcinoma.

TABLE 4. Differences in MVD between Solid and Nonsolid Adenocarcinoma Patients

Predominant Adenocarcinoma Subtypes	No. of Patients (%)	MVD		*p*
		Low[a]	High[b]	
Total	63	36 (57)	27 (43)	
Nonsolid subtypes	53 (84)	34 (64)	19 (36)	
Solid subtype	10 (16)	2 (20)	8 (80)	0.011[c]

Numbers in parentheses are in percentages.
[a] ≤median in the entire cohort.
[b] >median in the entire cohort.
[c] Significance.
MVD, microvessel density.

the most poorly differentiated among lung adenocarcinoma subtypes.[9,10] In addition, Ding et al.[11] reported that an EGFR mutation showed a significant positive correlation with BAC and papillary subtypes but not with the solid subtype. Thus, with the introduction of computed tomography screening and the discovery of targeted small molecule therapies against EGFR, there has been an enormous interest in the pathology, radiology, molecular biology, and clinical features of lung adenocarcinoma subtypes.

Several reports have revealed that angiogenesis plays a significant role in the pathogenesis of tumors and in the mechanisms of disease progression.[12,18] Recent reports have indicated that the transplantation of ex vivo cultivated EPCs reportedly contributes to angiogenic tumor vasculature.[29–31] Despite certain discrepancies in the existing reports,[32–34] the role of EPCs in vessel formation in tumors has now become widely accepted. Therefore, recent evidence demonstrating

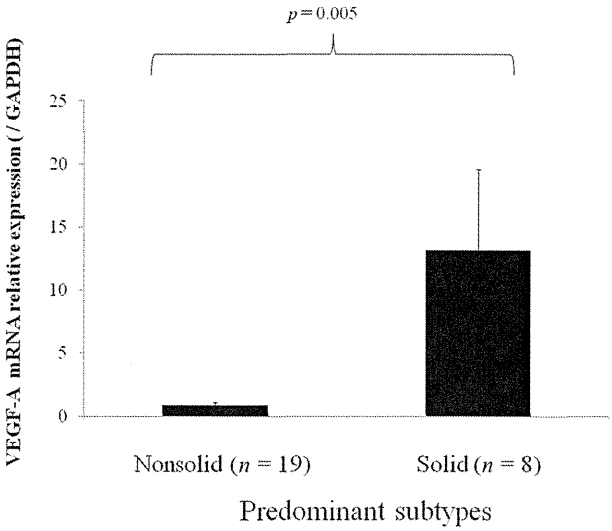

FIGURE 5. Differences in vascular endothelial growth factor (VEGF)-A levels in tumor tissue samples between solid and nonsolid adenocarcinoma patients, as determined using a quantitative real-time polymerase chain reaction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the existence of a bone marrow reservoir of EPCs and their selective involvement in neovascularization has attracted considerable interest because these cells could be used as surrogate markers to monitor the status of tumor angiogenesis.[16] In this study, the number of EPCs in patients with predominantly solid adenocarcinoma was significantly higher than in patients with predominantly BAC, papillary, or acinar adenocarcinomas. The higher number of circulating EPCs associated with solid adenocarcinoma may indicate the presence of differences in the tumor angiogenic status between early-stage adenocarcinoma histological subtypes.

In addition to the number of circulating EPCs, several reports have also demonstrated a significant correlation between neovascularization assessed using the intratumoral MVD, the tumor angiogenic status, and patient outcome in a variety of tumors.[17–19] In this study, the MVD of the tumor was significantly correlated with the number of circulating EPCs. Regarding the prognostic relevance of angiogenic activity in NSCLC as expressed by the intratumoral MVD, a high MVD has been identified as an unfavorable prognostic factor.[35,36] Yuan et al.[37] reported that the MVD was significantly correlated with the histological types and that a higher MVD was found significantly more frequently in adenocarcinoma than in squamous cell carcinoma, suggesting that adenocarcinomas might have a higher angiogenic potential. However, in this study, no statistically significant differences in the MVD were observed between adenocarcinoma and squamous cell carcinoma. Instead, we found significant differences in the MVD among the adenocarcinoma histological subtypes. A higher MVD was found significantly more frequently in solid adenocarcinomas than in nonsolid adenocarcinomas. This may reflect the aggressive and invasive characteristics of this subtype and may be one of the reasons why patients with solid adenocarcinoma have significantly poorer outcomes than those with other adenocarcinoma histological subtypes.

VEGF is the most important angiogenesis factor, and its expression within tumors is suggested to affect the prognosis of patients.[26,38] Thus far, there have been several reports regarding the association between the level of VEGF-A and MVD.[26,27,36] In addition, bone marrow-derived EPCs are also reported to be mobilized by the stimulation of tumor-derived VEGF-A, inducing them to migrate toward the tumor and to become incorporated into the developing neovasculature.[24,25] In this study, we confirmed that higher levels of VEGF-A are present in solid adenocarcinomas than in nonsolid adenocarcinomas. Recent studies have shown that the addition of antiangiogenic therapy, such as bevacizumab, to paclitaxel and carboplatin improves survival, compared with chemotherapy alone, in patients with previously untreated metastatic nonsquamous NSCLC.[13] Especially among adenocarcinoma patients, those with a solid adenocarcinoma may be the best candidates for the addition of bevacizumab, an antiangiogenic monoclonal antibody that blocks VEGF-A.

In this study, we showed a difference in the number of circulating EPCs or intratumoral MVD, both of which might be potential markers for neovascularization, between adenocarcinoma histological subtypes. Gao et al.[39] reported that circulating EPCs play a major and catalytic role in tumor progression, which may be maximized in metastatic and relapsing disease by the promotion of the progression of avascular micrometastases to vascularized macrometastases. The significantly higher levels of EPCs paralleling clinical severity also suggest the possible relevance of these cells in the metastatic progression of the tumors[16] and point to their potential use as targets in therapy against metastatic sites. Therefore, preoperative or postoperative anti-EPC therapy may be indicated for early-stage adenocarcinoma patients with preoperative high EPC levels to prevent postoperative recurrence after resection. In this study, the number of EPCs in patients with solid adenocarcinoma was significantly higher than that in nonsolid adenocarcinoma patients. This finding may indicate a subgroup of adenocarcinoma patients who may benefit from angiogenesis inhibitors targeted against EPCs.

This study had several limitations. In particular, the study lacked ethnic diversity, as all the patients were Japanese. Another limitation is that the blood mononuclear cells from the PA were isolated from the resected lungs and not directly from the patients preoperatively to avoid unnecessary invasiveness. However, we believe that the level of EPCs in the blood from the PA in the vicinity of the tumor more precisely reflects the effect of the tumors than the samples from the peripheral blood, as previously reported.[16,24] In this study, we first reported the differences in the number of circulating EPCs or MVD between lung adenocarcinoma subtypes. Further clinical studies are needed to confirm the beneficial effects of antiangiogenic therapy against VEGF or EPCs in solid adenocarcinoma patients.