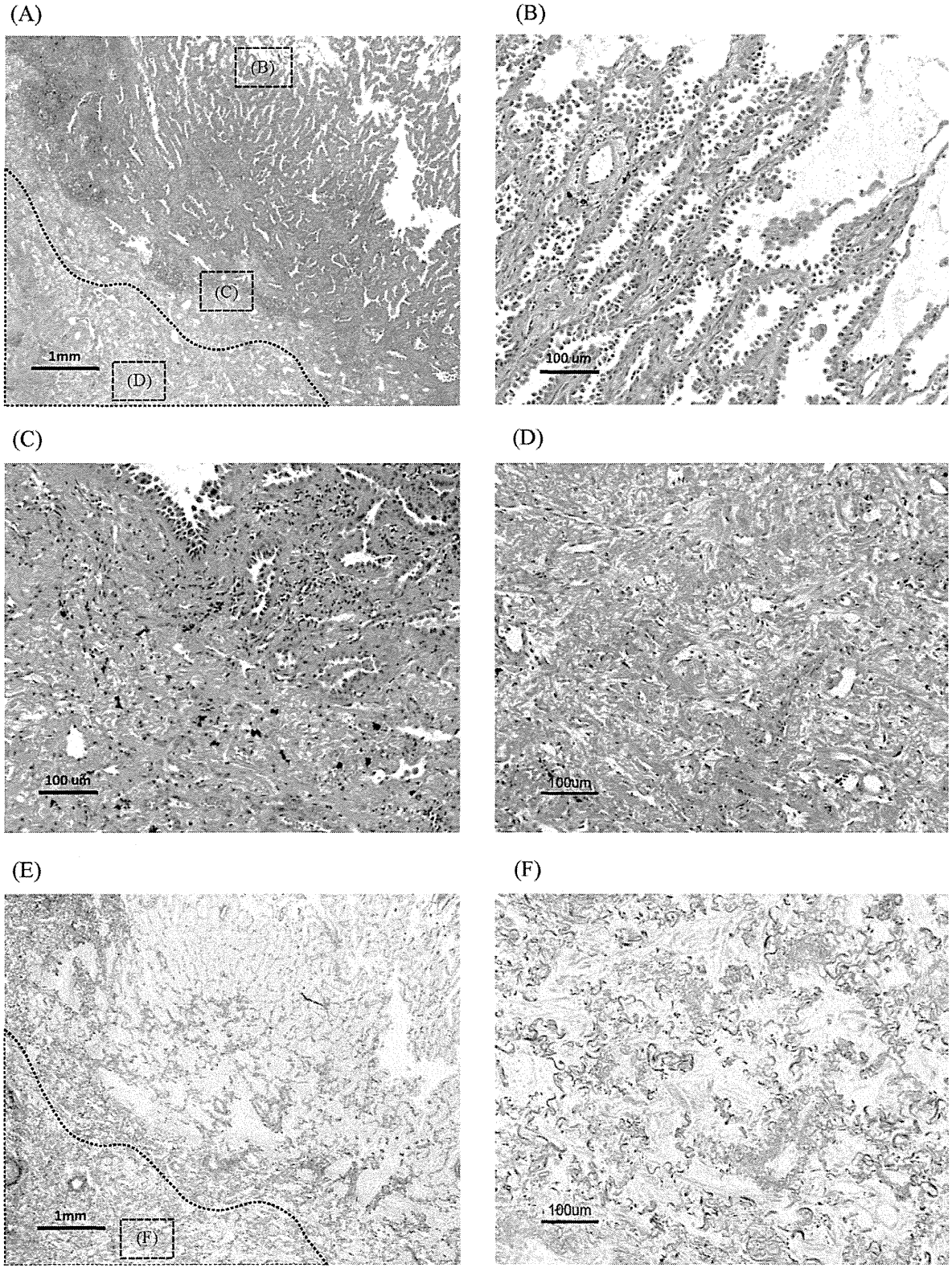

Fig. 1. Morphological features of lepidic growth (LG)-predominant type (A–E: hematoxylin and eosin staining, E: Victoria blue-Van Gieson (VVG) staining). (A) A lower magnification view of LG-predominant type adenocarcinoma. Dot-line represented non-cancerous cell collapse area (NCCA) as below. (B) LG component observed in the tumor periphery. (C) Interface between LG component and central collapse area. (D) NCCA: collapse area without cancer cells, which areas were separated from the closest cancer cells by greater than 1 mm. (E) A lower magnification view on VVG staining. (F) NCCA on VVG staining in higher magnification view.

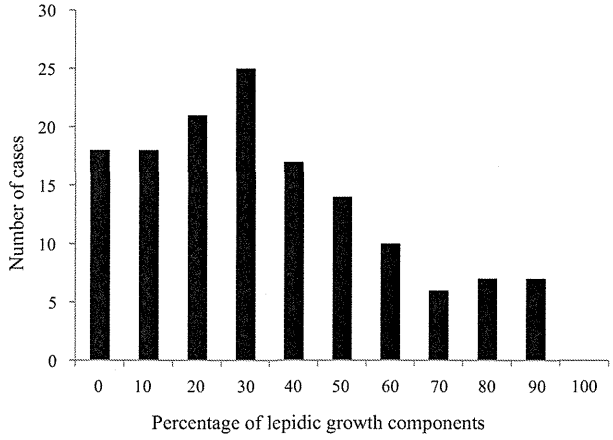

Fig. 2. Number of cases according to the percentage of lepidic growth components.

maximum axis on mediastinal window setting images. TDR was calculated by the following formula:

$$TDR = 1 - \frac{mDmax \times mDperp}{pDmax \times pDperp}$$

2.6. Statistical analysis

Statistical analysis was performed using SPSS software Version 11.0 (SPSS Inc., Chicago, IL). The length of overall survival (OS) was calculated in months from the date of surgery to the date of death from any cause or the date on which the patient was last known to be alive. Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test in univariate analysis. Recurrence-free survival (RFS) interval was calculated as the interval in months between the date of surgery and the date of recurrence, the date of death from any cause, or the most recent date on which the patient was last known to be alive. To determine independent prognostic factors, multivariate analysis was conducted using the Cox proportional hazard model. Two-category comparison was performed using the Pearson chi-squared test and the Fisher's exact test for quantitative data. NCCA and TDR were presented as mean ± standard deviation and were compared using Mann–Whitney *U* test. All tests were two-sided, and statistical significance was set at a *p*-value of less than 0.05.

3. Results

3.1. Univariate prognostic analysis according to the percentage of lepidic growth component

The median follow-up period of the surviving patients was 70.8 months. The distribution of cases according to the percentage of LG components is shown in Fig. 2. The mode percentage was 30%, and there were no cases with 100% LG components. We performed univariate prognostic analysis employing various percentage cut-offs of LG components in 10% increments. When the cut-off was set from 40% to 70%, significant prognostic difference was observed between cases below the cut-off and those above the cut-off, and the difference was most evident when the cut-off was set at 50% ($p < 0.001$, Supplementary Table 1). Therefore, we defined LG-predominant tumors as tumors with LG component occupying 50% or more of the entire tumor in the following analyses.

Supplementary data related to this article found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2012.10.013.

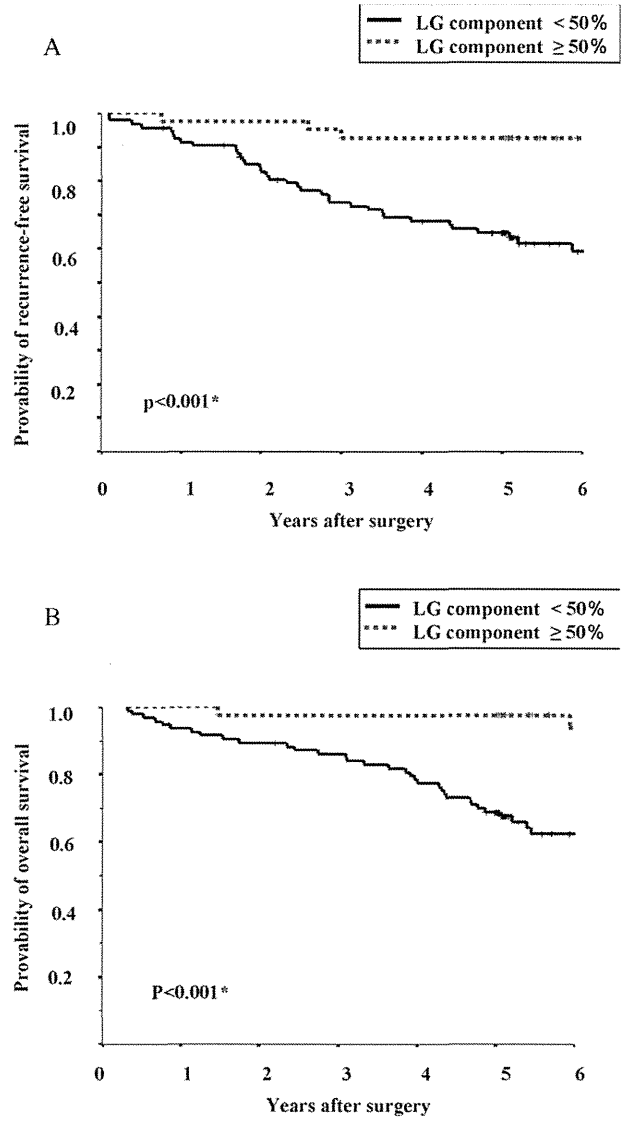

Fig. 3. (A) Recurrence-free survival curves of the non-LG-predominant type and LG-predominant type. (B) Overall survival curves of the non-LG-predominant type and LG-predominant type. LG, lepidic growth, *, log-rank test.

Fig. 3A shows the RFS curves according to the dominance of LG components (non-predominant type vs. predominant type). The 5-year RFS rates of the non-LG predominant type and LG-predominant type were 61.7% and 91.3%, respectively. The RFS of the LG-predominant group was significantly better than that of the non-LG -predominant group ($p < 0.001$). Fig. 3B shows the OS curves according to the dominance of LG components. The 5-year OS rates of the patients with non-LG -predominant type and LG-predominant type were 65.0% and 93.5%, respectively. The OS of the LG-predominant group was significantly better than that of the non-LG -predominant group ($p < 0.001$).

3.2. Clinicopathological characteristics of the lepidic growth-dominant tumors

We compared the clinicopathological characteristics of the LG-predominant and non-LG-predominant groups (Table 1). Female gender ($p = 0.039$), smoking history of <20 pack-years ($p = 0.039$), absence of pleural invasion ($p = 0.003$), and absence of

Table 1
Relationship between percentages of lepidic growth components and clinicopathological characteristics.

Factors	Percentage of LG components		*p*-Value[a]
	≥50%	<50%	
Age (years)			
<68	25 (61.0%)	43 (45.7%)	
≥68	16 (39.0%)	51 (54.3%)	0.134
Gender			
Male	16 (39.0%)	56 (59.6%)	
Female	25 (61.0%)	38 (40.4%)	0.039
Smoking history (pack-years)			
<20	29 (70.7%)	48 (51.1%)	
≥2012 (29.3%)	46 (48.9%)	0.039	
Preoperative CEA (mg/dL)			
<5.0	28 (68.3%)	47 (50.0%)	
≧5.0	13 (31.7%)	47 (50.0%)	0.060
Lymphatic permeation			
Absence	34 (82.9%)	65 (69.1%)	
Presence	7 (17.1%)	29 (30.9%)	0.138
Vascular invasion			
Absence	36 (39.0%)	38 (40.4%)	
Presence	5 (39.0%)	56 (59.6%)	<0.001
Pleural invasion			
Absence	34 (82.9%)	53 (56.4%)	
Presence	7 (17.1%)	41 (43.6%)	0.003

LG, lepidic growth.

[a] Fisher's exact test, CEA, serum carcinoembryonic antigen level.

vascular invasion ($p < 0.001$) were significantly more frequently observed in the LG-predominant group.

We evaluated NCCA (Fig. 1A and E) as its percentage to the maximum cut-surface area. The LG-predominant tumors showed significantly higher percentages ($29.4 \pm 6.1\%$ vs. $5.0 \pm 1.9\%$, $p < 0.001$) (Fig. 4A).

As shown in Fig. 4B, TDR on chest CT images for LG-predominant type and non-LG-predominant type were 0.339 ± 0.189 and 0.293 ± 0.277, respectively. There were no significant differences between the two groups ($p = 0.168$).

3.3. Multivariate analysis

We performed multivariate analysis to determine independent prognostic factors, including the following factors as covariates: age, gender, smoking history, preoperative CEA, pleural invasion, vascular invasion, lymphatic permeation, and percentage of LG components. Absence of pleural invasion (HR = 0.243, 95% confidence interval (CI): 0.082–0.711, $p = 0.001$), absence of vascular invasion (HR = 0.511, 95% CI: 0.268–0.974, $p = 0.041$), and LG-predominant (LG component ≥ 50%) type (HR = 0.285, 95% CI: 0.148–0.574, $p = 0.014$) were found to be independent favorable prognostic factors (Table 2).

4. Discussion

In the current study, we focused on the LG components in node-negative adenocarcinomas 3–5 cm in size. Several previous reports have described that the proportion of LG components is related to tumor aggressiveness and prognosis in small adenocarcinomas less than 3 cm in size [5,10–12]. There are also a few previous reports describing the prognostic significance of LG components in adenocarcinomas greater than 3 cm in size. Mizuno et al. reported that LG-predominant histology was a significant prognostic factor among patients with adenocarcinomas which were diagnosed as pathological stage IB, according to the 6th edition TNM classification [13]. Their study subject included node-negative tumors that were (1) greater than 3 cm in diameter and/or (2) invading to the visceral pleura surface [14]. Although they identified absence of pleural invasion, absence of vascular invasion, and LG-predominant histology as favorable prognostic indicators, their study subject was highly heterogeneous. For example, a tumor of 7 cm in size with pleural surface invasion and a tumor 3.1 cm in size without pleural invasion were included and collectively evaluated. In the current study, we aimed at analyzing a relatively homogeneous cohort by limiting the study subject only to tumors of 3–5 cm in size without node involvement.

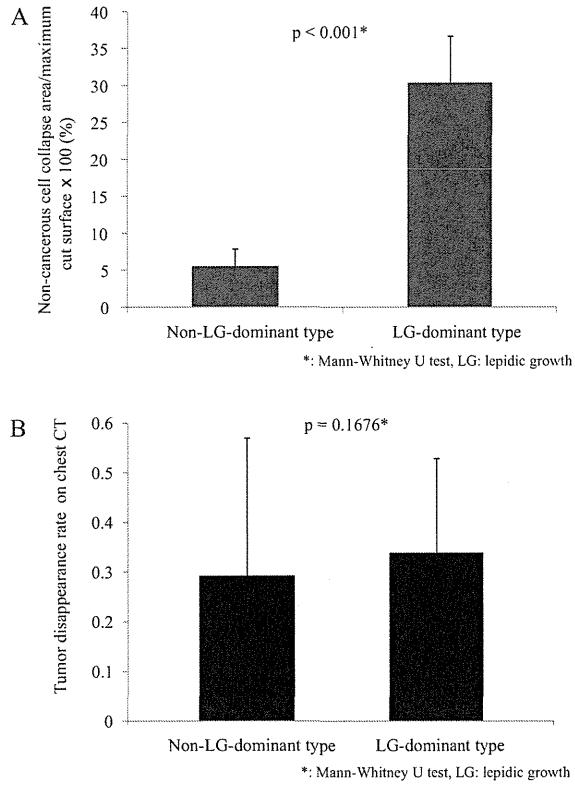

Fig. 4. (A) Percentages of non-cancerous cell collapse area (NCCA) according to lepidic growth component predominance. (B) Tumor disappearance rate (TDR) on chest CT images according to lepidic growth component predomoinance.

In our study, the LG-predominant type was an independent favorable prognostic indicator and correlated significantly with absence of vascular and pleural invasion. In the center of the tumor, these LG-predominant tumors had significantly larger NCCA compared with non-LG-predominant tumors. Non-invasive (bronchioloalveolar) adenocarcinoma is generally speculated to progress gradually into invasive adenocarcinoma, in which central fibrosis is usually observed histologically [5,15]. Central fibrosis is reported to be associated with invasive process and cancer cell invasion in the central fibrotic focus has been reported in many cases [15–17]. However, LG-predominant type tumors often have larger NCCA in the center, and these collapse areas are free from cancer cells. NCCA in LG-predominant tumors may develop through a pathway different from that of central fibrotic foci. And we also considered that favorable prognosis of LG-dominant type cases possibly related to having large NCCA in some way.

It has been reported that the cancer cell invasion of visceral pleura was correlated with poor prognosis in many literatures [18,19]. According to our result, small NCCA may be a phenotype reflecting local invasiveness of cancer cells along with pleural invasion and central fibrotic foci. Correlation between the

Table 2
Multivariate prognostic analysis.

Variables	Favorable	Unfavorable	Hazard ratio	95% CI	*p*-Value
Age (years)	<68	≥68	0.812	0.350–1.887	0.629
Gender	Female	Male	0.821	0.443–1.522	0.531
Smoking history (pack-years)	<20	≥20	0.546	0.270–1.104	0.084
Preoperative CEA (mg/dL)	<5.0	≥5.0	0.843	0.451–1.575	0.592
Lymphatic permeation	Absence	Presence	0.754	0.427–1.331	0.330
Pleural invasion	Absence	Presence	0.243	0.082–0.711	0.001
Vascular invasion	Absence	Presence	0.511	0.268–0.974	0.041
LG component (%)	≥50	<50	0.285	0.148–0.574	0.014

CI, confidence interval, CEA, serum carcinoembryonic antigen level, LG, lepidic growth.

morphological and molecular features and proportion of NCCA will be required in the further study.

In previous reports, adenocarcinoma associated with features of LG pattern is known to have frequent epidermal growth factor receptor (EGFR) mutations and potentially sensitive to EGFR-tyrosine kinase inhibitors [20–23]. In contrast, Ninomiya et al. reported that no correlation between LG predominant histology and EGFR mutation were shown [24]. We considered that additional information about correlations between EGFR mutation and histological features would be valuable. Regrettably, however, because we had not performed EGFR mutation screening in practice routinely until 2004 in our institution, it is unable to do research related to EGFR mutation in the current cohort. It is really interesting and we are now making arrangements to investigate this point.

A typical CT finding corresponding to LG components is ground-glass attenuation, which is hazy increased attenuation of the lung but with preservation of bronchial and vascular margins [25]. In contrast, the other components are usually depicted as solid attenuation. Accordingly, LG-predominant type tumors are likely to appear as mixed ground-glass attenuation nodules on CT, as previously described [26–29]. Lee et al. reported that TDR on CT correlated well with the proportion of LG components and that lower TDR was an independent risk factor for poor prognosis in patients with small-sized lung adenocarcinoma [30]. In the current study, we compared TDR in the histologically most representative 20 cases of each of the 2 groups according to the proportion of LG components (LG-predominant type vs. non-LG-predominant type). However, no significant difference was observed between the groups. This may be because NCCA in LG-predominant tumors are depicted as consolidation on CT, similarly to central fibrosis in invasive adenocarcinomas. Therefore, it was difficult to estimate LG-predominance correctly based on TDR on preoperative high-resolution CT. Furthermore, we presume that TDR is not a prognostic indicator because the TDR was not able to reflect the LG-predominant tumors with large NCCA correctly. However, we have to note that we could not include TDR in survival analysis in the same way as the other factors, for example LG component, because we performed TDR analysis in only 20 cases of each groups in which we could get high-resolution CT.

There were several limitations in the current study. This was a retrospective study carried out at a single institution on an ethnically homogenous population. The total number of cases was relatively small. However, our data clearly demonstrated that LG component predominance was significantly associated with distinctive clinicopathological features and favorable prognosis.

In conclusion, we demonstrated that LG-predominant type tumors had less invasiveness and greater NCCA in adenocarcinomas without node involvement 3–5 cm in size. Further investigation of the biology of this type of adenocarcinoma may provide new insight into tumor progression mechanism.

Funding

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Conflicts of interest

All authors declare that they have no conflicts of interest regarding this manuscript.

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ORIGINAL ARTICLE

The clinical outcome of non-small cell lung cancer patients with adjacent lobe invasion: the optimal classification according to the status of the interlobar pleura at the invasion point

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Abstract

OBJECTIVES: The aim of this study was to analyse the survival of non-small cell lung cancer (NSCLC) patients with adjacent lobe invasion (ALI) with emphasis on the interlobar fissure status at the tumour invasion point.

METHODS: We retrospectively evaluated 2097 consecutive patients with surgically resected NSCLC from July 1993 through April 2006. Of these, 90 (4.3%) patients had tumours with ALI. We divided ALIs into two types by histological examination using elastic stains: direct ALI beyond the incomplete fissure (ALI-D, $n = 18$) and ALI across the interlobar fissure (ALI-A, $n = 72$), and compared the clinicopathological features and survival.

RESULTS: The patients with ALI demonstrated an intermediate survival between T2a and T2b tumours (5-year overall survival: T2a, 61.0%; ALI, 59.6%; T2b, 49.2%). There were distinct survival differences between the patients with ALI-A and ALI-D (5-year overall survival: ALI-D, 85.7%; ALI-A, 52.0%; $P = 0.010$). The survival of patients with ALI-A was not statistically different from that of patients with T2b tumours, regardless of the tumour size ($P = 0.846$). The survival of the patients with ALI-D did not statistically differ from those with T1a or T1b tumours ($P = 0.765$ and 0.418, respectively).

CONCLUSIONS: Our results indicate that the interlobar fissure status affects the survival of the patients with ALI. ALI should be examined by elastic stains and only ALI-A should be classified as true ALI. We propose that ALI-A tumours with a size of ≤5 cm should be assigned to T2b, but ALI-D tumours do not require an adjustment of the T descriptor.

Keywords: Non-small cell lung cancer • Pleural invasion • Adjacent lobe invasion • Interlobar pleural invasion • Interlobar fissure

INTRODUCTION

Visceral pleural invasion (VPI) has been reported as one of the most important prognostic factors in non-small cell lung cancer (NSCLC) patients who have undergone surgical resection [1–3]. Furthermore, it has been reported that tumour invasion of an adjacent lobe across the interlobar pleura affected the outcome of patients with NSCLC [4–8]. Adjacent lobe invasion (ALI) was first defined as T2 in the 6th TNM classification of the Union for International Cancer Control (UICC). In the 7th TNM classification of the UICC, [9] NSCLCs with ALI are classified as T2a unless other criteria assign a higher T category, regardless of whether they are across the complete fissure or the incomplete fissure. However, no validation analyses have been conducted on the prognostic impact of ALI, and furthermore, the TNM staging system does not consider the fissure status at the tumour invasion point. In this retrospective study, we analysed the survival of NSCLC patients with ALI and validated the definition of ALI in the 7th TNM classification.

MATERIALS AND METHODS

Patients

From July 1993 through April 2006, 2471 consecutive patients underwent surgical resection for a primary non-small cell lung carcinoma at our institution. We examined the data of a total of 2097 patients who underwent complete resection by at least lobectomy and systematic lymph node dissection. The remaining 374 patients were excluded from this study (Fig. 1). Data collection and analyses were approved, and the need to obtain informed consent from each patient was waived by the institutional review board in December 2010.

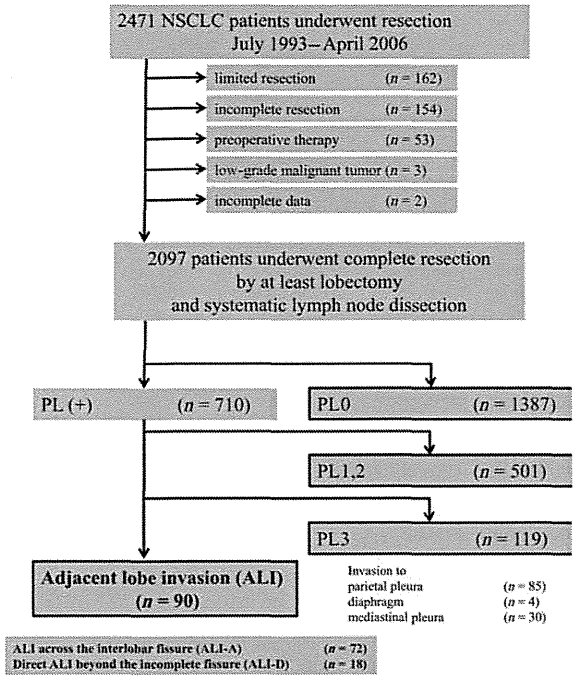


Figure 1: Patients enrolled in the study.

The clinicopathological characteristics of the study population are summarized in Table 1. The median follow-up period was 84 months (range, 1–178 months). Modes of resection were pneumonectomy in 107 patients (5%), bilobectomy in 108 patients (5%), lobectomy plus wedge resection or segmentectomy in 165 patients (8%) and lobectomy in 1717 patients (82%). Adenocarcinoma was the most common histological type (68%).

Among 2097 patients, 710 patients had tumours with pleural invasion including ALI. In this study, we first defined tumours with ALI in accordance with the 7th TNM classification, [9] disregarding the status of the interlobar pleura at the invasion point. A total of 90 ALI tumours were extracted from the tumours with VPI (PL1 or 2, $n = 501$) and from the tumours with invasion of the parietal pleura or other structures except an adjacent lobe (PL3, $n = 119$) (Fig. 1). We then classified the ALI tumours into two types, defined as either the tumours directly extending into the adjacent lobe beyond the incomplete fissure point (ALI-D, $n = 18$; Fig. 2A) or the tumours with ALI across the complete interlobar fissure (ALI-A, $n = 72$; Fig. 2B), and compared the clinicopathological features of each group. We categorized the tumour with a combination of ALI-D and ALI-A as an ALI-A tumour, and we classified the tumours invading the adjacent lobe beyond only incomplete fissure points as ALI-D tumours.

Clinicopathological information collection

We reviewed the medical records of each patient for clinicopathological information, including age, gender, smoking history, preoperative serum carcinoembryonic antigen (CEA) level (cut-off at the normal upper limit of 5 ng/ml), mode of resection, histology, tumour size, pathological nodal status, lymphatic permeation, vascular invasion and pleural invasion as defined in the TNM Classification, 7th edition [1, 2].

Table 1: Patient characteristics

Characteristics	Number of patients (%) (*n* = 2097)
Age	
Median (years)	65
Range	(20–89)
Gender	
Men	1323 (63)
Women	774 (37)
Smoking history	
Never	674 (32)
Yes	1423 (68)
CEA (ng/ml)	
<5.0	1239 (59)
≥5.0	858 (41)
Mode of resection	
Pneumonectomy	107 (5)
Bilobectomy	108 (5)
Lobectomy + wedge resection or segmentectomy	165 (8)
Lobectomy	1717 (82)
Histology	
Adenocarcinoma	1422 (68)
Squamous cell carcinoma	470 (22)
Large cell carcinoma	58 (3)
Adenosquamous carcinoma	54 (2)
LCNEC	34 (2)
Others	59 (3)
Tumour size (cm)	
Mean ± SD	3.4 ± 1.9
Pathological nodal status	
pN0	1483 (71)
pN1	315 (15)
pN2	299 (14)
Lymphatic permeation	
Negative	1409 (67)
Positive	688 (33)
Vascular invasion	
Negative	1024 (49)
Positive	1073 (51)

LCNEC: large cell neuroendocrine carcinoma.

Histological examination of pleural or interlobar invasion

All surgical specimens were fixed with 10% formalin and embedded in paraffin. The tumours were cut in the horizontal section in accordance with a computed tomography (CT) scan at ~5-mm intervals including the maximum section of the tumour and macroscopically the most invasive part of the pleura to examine tumour invasion of the adjacent lobe, and serial 4-μm sections were stained with haematoxylin and eosin. The Victoria blue-van Gieson method was used to visualize the elastic fibres. Blood and lymphatic vessels were identified by haematoxylin–eosin and Victoria blue-van Gieson stains, and vascular invasion and lymphatic permeation were histologically diagnosed by identifying cancer cells within blood and lymphatic vessels, respectively. Histological diagnoses were based on the revised 3rd edition of the World Health Organization Classification of Tumours [10]. Pathological T classification and N classification were determined based on the 7th TNM Classification for Lung and Pleural Tumours of the UICC [11]. Pleural invasion was prospectively evaluated with the aid of the Victoria blue-van Gieson method by

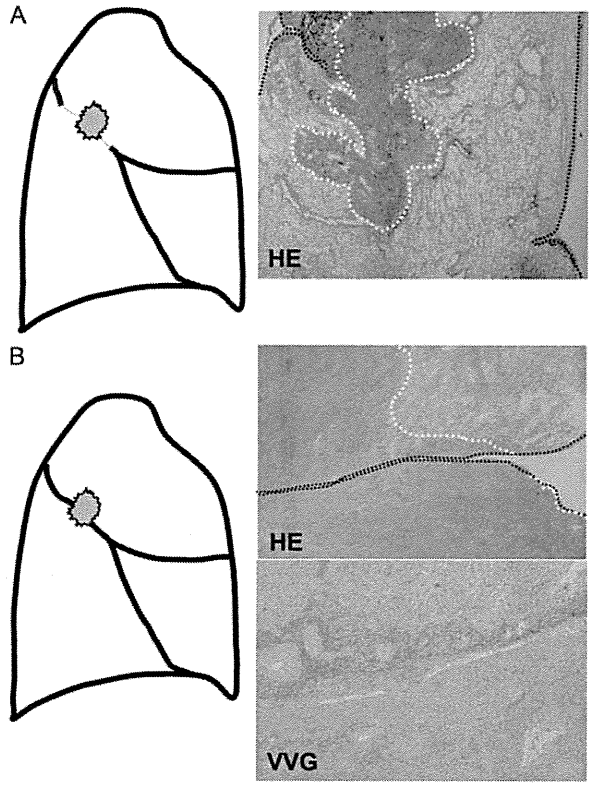


Figure 2: Two patterns of ALI of the tumour are shown. ALI-D, direct tumour (yellow dots) extension into the adjacent lobe beyond the point where the interlobar fissure (black dots) is deficient (**A**); and ALI-A, tumour (yellow dots) invasion into an adjacent lobe across the interlobar fissures (black dots) (**B**).

more than two pathologists. Each pathological specimen was also re-examined by two pathologists who were blinded to the clinical outcome (Y.O. and G.I.) for this study, and the interlobar fissure status and the degree of pleural invasion and ALI were determined.

Patient follow-up

We examined the 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 pertinent tumour markers. Whenever any symptoms or signs of recurrence were detected, further evaluations including CT scans of the chest and abdomen, brain magnetic resonance imaging and bone scintigraphy were performed. Since 2004, integrated positron emission tomography and CT have also been performed for selected patients.

Statistical analysis

The overall survival (OS) was defined as the time interval between the date of surgery and the date of death from any cause. The last follow-up observation was censored when the patient was alive or lost to follow-up. To compare the outcomes of ALI tumours with non-ALI tumours with other T factors, we selected ALI cases

Table 2: Characteristics of patients with ALI according to the invasion pattern

	Number of patients (%)		*P*-value
Interlobar fissure status in adjacent lobe invasion point	ALI-A (*n* = 72)	ALI-D (*n* = 18)	
Age			
Median (years)	67	67	0.175†
Range	39–86	59–79	
Gender			
Men	52 (72)	7 (39)	0.008*
Women	20 (28)	11 (61)	
Smoking history			
Never	13 (18)	6 (33)	0.155*
Yes	59 (72)	12 (67)	
CEA (ng/ml)			
<5.0	30 (42)	9 (50)	0.523*
≥5.0	42 (58)	9 (50)	
Mode of resection			
Pneumonectomy	14 (20)	2 (11)	0.408[a],*
Bilobectomy	19 (26)	3 (17)	
Lobectomy + wedge resection	36 (50)	9 (50)	
Lobectomy + segmentectomy	3 (4)	4 (22)	
Main location and invasion lobe			
RUL and RML	24 (33)	6 (33)	0.907[b],*
RML and RLL	11 (15)	2 (11)	
RUL and RLL	16 (22)	5 (28)	
LUL and LLL	21 (29)	5 (28)	
Histology			
Adenocarcinoma	40 (56)	12 (66)	0.393[c],*
Squamous cell carcinoma	23 (32)	3 (17)	
Large cell carcinoma	6 (8)	3 (17)	
Adenosquamous carcinoma	3 (4)	0 (0)	
Tumour size (cm)			
Mean ± SD	4.6 ± 1.8	4.2 ± 1.2	0.290†
≤2.0	4 (5)	0 (0)	
>2.0–3.0	10 (14)	2 (11)	
>3.0–5.0	35 (49)	12 (67)	
>5.0–7.0	16 (22)	4 (22)	
>7.0	7 (10)	0 (0)	
Pathological nodal status			
pN0	26 (36)	6 (33)	0.030[d],*
pN1	23 (32)	11 (61)	
pN2	23 (32)	1 (6)	
Lymphatic permeation			
Negative	30 (42)	11 (61)	0.138*
Positive	42 (58)	7 (39)	
Vascular invasion			
Negative	9 (12)	7 (39)	0.009*
Positive	63 (88)	11 (61)	
Recurrence	40 (56)	5 (28)	
Loco-regional	9 (13)	3 (17)	–
Distant	18 (25)	2 (11)	
Loco-regional and distant	13 (18)	0	
Five-year overall survival rate	49.8	76.6	0.009‡

RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LUL: left upper lobe; LLL: left lower lobe.
[a]Pneumonectomy vs the others.
[b]Right side vs left side.
[c]Adenocarcinoma vs non-adenocarcinoma.
[d]pN0 vs pN1 vs pN2.
*Pearson's χ^2 test or Fisher's exact test.
†Independent samples *t*-test.
‡Log-rank test.

without any other criteria higher than T2b category, i.e. <5 cm maximum diameter and no evidence of pulmonary metastasis. The correlations between the lobulation status in the section showing tumour invasion and the clinicopathological factors were evaluated by the χ^2 test, the Fisher exact test or the Student *t*-test. For univariate analyses, all cumulative OS rates were estimated by the Kaplan–Meier method, and survival differences were compared using the log-rank test. Multivariate analyses were performed using the Cox proportional hazard model. Forward and backward stepwise procedures were performed to determine the combination of prognostic factors. All *P*-values reported are two-sided, and the significance level was set at below 0.05. Analyses were performed with SPSS 11.0 statistical software (Dr. SPSS II for Windows, standard version 11.0, SPSS Inc., Chicago, IL, USA).

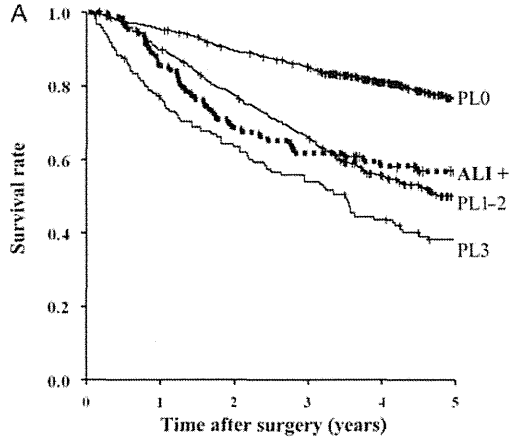
RESULTS

Clinicopathological features of the patient with ALI

The characteristics of the patients with ALI tumours ($n = 90$) are shown according to the type of ALI in Table 2. There were significantly more men and more tumours with pN2 disease and vascular invasion in the ALI-A group than the ALI-D group. Although the mean tumour sizes did not significantly differ between the two groups, large-sized tumours of more than 7 cm were observed only in the ALI-A group.

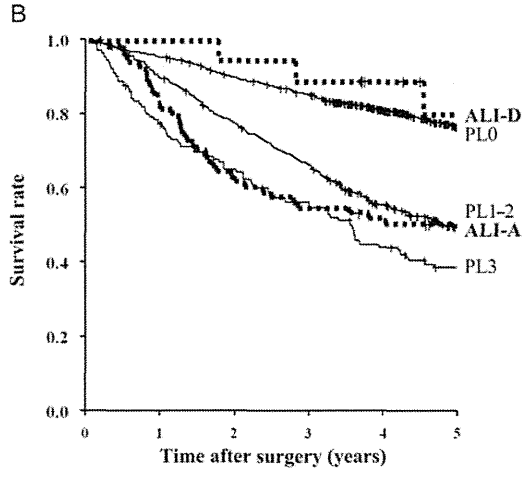
Survival of the patients with adjacent lobe invasion or visceral pleural invasion

Twenty-nine patients (40%) in the ALI-A group and five patients (28%) in the ALI-D group had recurrences during the post-operative follow-up. The ALI-A group developed nine (13%) loco-regional recurrences including five intrapulmonary metastases, one recurrence in the mediastinal lymph node, one pleural dissemination and two multiple recurrences in the thoracic cavity. In the ALI-D group, three (17%) loco-regional recurrences were detected: two intrapulmonary metastases and one pleural dissemination. No patients developed recurrence in the resected surgical margin in both groups. The OS rates of the patients with ALI and those with various degrees of VPI are shown in Fig. 3A.



		No. of patients	5-year OS (%)
PL0		1387	76.6
PL1-2		501	49.8
PL3		119	37.9
ALI		90	56.2
			P-value※
PL0	vs.	Others	<0.001
ALI	vs.	PL1-2	0.395
		PL3	0.035
PL1-2	vs	PL3	0.040

※*p*-value by log-rank test.



		No. of patients	5-year OS (%)
ALI-D		18	76.6
ALI-A		72	49.8
			P-value※
ALI-D	vs.	PL0	0.507
		PL1-2	0.008
		PL3	0.002
ALI-A	vs.	PL0	<0.001
		PL1-2	0.527
		PL3	0.456
ALI-A	vs.	ALI-D	0.009

※*P*-value by log-rank test. ALI-A: adjacent lobe invasion - across the interlobar fissure, ALI-D: adjacent lobe invasion - direct beyond the incomplete fissure.

Figure 3: The overall survival rates of NSCLC patients with ALI and with various degrees of VPI. The patients with ALI showed significantly better survival than those with PL3 tumours ($P = 0.035$) and demonstrated the survival overlapped with those with PL1–2 tumours ($P = 0.395$) (**A**). The patients with ALI-D showed a significantly better survival than those with PL1–2, PL3 and ALI-A tumours ($P = 0.008$, 0.002 and 0.009, respectively). The survival of the patients with ALI-D did not statistically differ from that of the patients with PL0 tumours ($P = 0.507$) (**B**).

Patients with ALI showed significantly worse survival than those with PL0 and better survival than those with PL3 (T3) tumours ($P < 0.001$ and 0.035, respectively). The survival of the patients with ALI was statistically not different from that of the patients with PL1-2 tumours ($P = 0.395$). Figure 3B shows the OS curves of the patients with the two different types of ALIs: ALI-A and ALI-D. The patients with ALI-D tumours showed significantly better survival than those with PL1-2, PL3 and ALI-A tumours ($P = 0.008$, 0.002 and 0.009, respectively). The survival of patients with ALI-D tumours did not differ statistically from that of the patients with PL0 tumours ($P = 0.507$).

Prognostic factors of the patients with adjacent lobe invasion

Univariate analyses of the patients with ALI tumours for OS are shown in Table 3. In the entire study cohort, significant survival differences were observed according to the pathological N status, vascular invasion status and type of ALI ($P = 0.007$, 0.001 and 0.009, respectively). Multivariate analysis revealed that the presence of vascular invasion and pN2 status were statistically significantly worse prognostic factors for the patients with an ALI tumour [vascular invasion, hazard ratio (HR): 4.64, 95% confidence interval (CI): 1.06-20.24, $P = 0.041$; pN2, HR: 2.17, 95% CI: 1.01-4.65, $P = 0.047$). However, the type of ALI also tended to influence survival, although the difference was not statistically significant. The patients with ALI-A had a trend towards more risk of death relative to those with ALI-D (HR: 2.47, 95% CI: 0.85-7.17, $P = 0.097$).

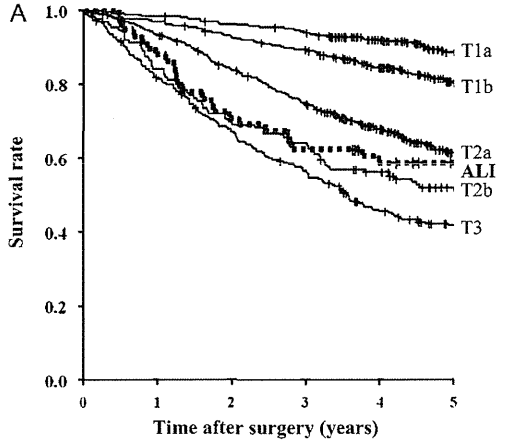
Assortment of adjacent lobe invasion in the T classification of the 7th TNM staging system

The OS curves of the patients stratified by pathological T factors up to T3 and ALI are shown in Fig. 4A. In total, 60 patients (36 men and 24 women) had an ALI tumour without any other criteria higher than the T2b category and were enrolled in this analysis. The survival differences between patients with ALI tumours

Table 3: Prognostic factors of the patients with ALI for overall survival

Characteristics	*n*	5-year OS	Univariate analysis, *P*-value†	Multivariate analysis	
				HR (95% CI)	*P*-value‡
Age (years)					
<67	43	51.4	0.655		
≥67	47	60.6			
Gender					
Male	59	56.2	0.946		
Female	31	54.2			
Smoking history					
Never	19	52.6	0.654		
Yes	71	56.3			
CEA (ng/ml)					
<5.0	39	62.3	0.154		
≥5.0	51	50.3			
Mode of resection					
Pneumonectomy/bilobectomy	38	50.9	0.327		
Lobectomy + wedge resection or segmentectomy	52	59.0			
Histology					
Adenocarcinoma	52	49.1	0.280		
Non-adenocarcinoma	38	64.9			
Tumour size (cm)					
≤3.0	16	49.2	0.968		
>3.0	74	56.9			
Pathological nodal status					
pN0	32	71.4	0.007§	1.00	0.109
pN1	34	58.3		1.46 (0.68-3.13)	0.328
pN2	24	29.8		2.17 (1.01-4.65)	0.047§
Lymphatic permeation					
Negative	41	61.5	0.352		
Positive	49	50.0			
Vascular invasion					
Negative	16	93.8	0.001§	1.00	0.041§
Positive	74	47.0		4.64 (1.06-20.24)	
Type of ALI					
ALI-D	18	76.6		1.00	0.097
ALI-A	72	49.8	0.009§	2.47 (0.85-7.17)	

†Log-rank test.
‡Cox's proportional hazard model.
§Denotes significance.

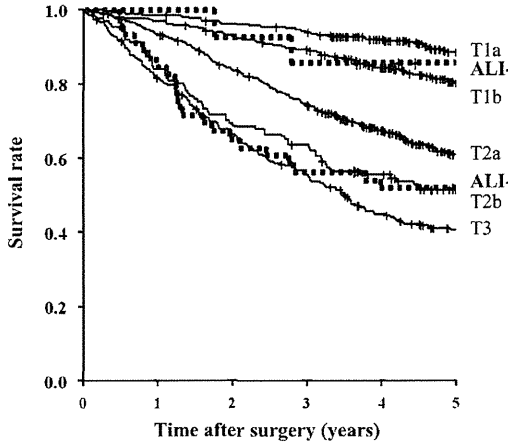


	No. of patients	5-year OS (%)
T1a	451	88.5
T1b	419	80.2
T2a	696	61.0
T2b	140	49.2
T3	322	41.1
ALI※	60	59.6

※Without any other criteria higher than T2b category. 5-year OS :5-year overall survival.

			P-value※
ALI	vs.	T1a	<0.001
		T1b	<0.001
		T2a	0.381
		T2b	0.408
		T3	0.042

※ *P*-value by log-rank test.



	No. of patients	5-year OS (%)
ALI-D※	14	85.7
ALI-A※	46	52.0

※Without any other criteria higher than T2b category. 5-year OS: 5-year overall survival.

			P-value※
ALI-D	vs.	T1a	0.765
		T1b	0.418
		T2a	0.040
		T2b	0.009
		T3	0.002
ALI-A	vs.	T1a	<0.001
		T1b	<0.001
		T2a	0.045
		T2b	0.846
		T3	0.358
ALI-A	vs.	ALI-D	0.010

※*P*-value by log-rank test. ALI-A: adjacent lobe invasion—across the interlobar fissure; ALI-D: adjacent lobe invasion—direct beyond the incomplete fissure.

Figure 4: The overall survival rates of NSCLC patients with ALI and with various degrees of pathological T classification. The survival of patients with ALI was between those of patients with T2a tumours and T2b tumours (**A**). Although the survival of the patients with ALI-D did not statistically differ from that of the patients with T1 tumours, it was significantly better than survival in those with tumours with a T factor of T2a or greater. The patients with ALI-A showed significantly worse survival than those with T2a tumours or ALI-D tumours ($P = 0.045$ and 0.010, respectively). The survival of patients with ALI-A did not statistically differ from patients with T2b tumours ($P = 0.846$) (**B**).

and those with tumours with T1a, T1b or T3 factors were statistically significant ($P < 0.001$, <0.001 and 0.042, respectively). The survival curve of patients with ALI tumours almost overlapped that of patients with T2b tumours ($P = 0.408$) and was also not different from patients with a T2a tumour ($P = 0.381$).

Figure 4B shows the survival curves stratified by pathological T factors up to T3 and two types of ALIs: ALI-A ($n = 46$) and ALI-D ($n = 14$). The survival of the patients with ALI-D tumours was significantly better than that of ALI-A tumours (5-year OS: ALI-D, 85.7%; ALI-A, 52.0%; $P = 0.010$). The patients with ALI-D tumours showed significantly better survival than those with a T factor of T2a or greater. The survival of the patients with ALI-D was statistically not different from that of the patients with T1a or T1b tumours ($P = 0.765$ and 0.418, respectively), although almost all ALI-D tumours were more than 3 cm. On the other hand, the patients with ALI-A tumours had significantly worse outcomes than those with T2a tumours ($P = 0.045$). The survival of the patients with ALI-A was statistically not different from that of the patients with T2b tumours ($P = 0.846$).

DISCUSSION

ALI has been recognized as one of the prognostic factors for resected NSCLCs. Several researchers have reported on the survivals of NSCLC patients with ALI and have tried to incorporate the ALI status into the 6th TNM classification, as shown in Table 4. Some investigators reported that the prognosis of ALI tumours was equivalent to that of T2 tumours, [4, 6], while others demonstrated that it was similar to the prognosis of T3

Table 4: The survival of NSCLC with ALI in the literature

Study	Author (year)	No. of patients	Frequency of ALI, *n* (%)	ALI type (%)	5-year OS (%)	Recommended T-factor in TNM classification
1	Miura (1998)	NA	18 (NA)	ALI-A (100%)	34	T2[a]
2	Okada (1999)	901	19 (2)	ALI-A (100%)	37	T3[a]
3	Nonaka (2005)	322	50 (15)	ALI-A (100%)	63	T2[a] (only Sq)
4	Demir (2007)	351	60 (17)	ALI-A (100%)	36	T3[a]
5	Yang (2009)	2094	28 (1)	ALI-A (100%)	41	T3[1]
6	Present study	2097	90 (4)	–	56	T2a or T2b[b]
	ALI-A		72 (3)	ALI-A (80%)	50	T2b[b]
	ALI-D		18 (1)	ALI-D (20%)	77	Not upstaging

Sq: squamous cell carcinoma.
[a]6th TNM.
[b]7th TNM.

tumours [5, 7, 8]. The categorization of NSCLCs with ALI may result in a prognosis between that of T2 and T3 tumours in the 6th TNM classification. In the 7th TNM classification, NSCLCs with ALI are classified as T2a, regardless of the fissure status at the site of the tumour invasion. We often encounter lobes with incomplete interlobar fissures. Although the new TNM staging system does not consider the fissure status in the diagnosis of ALI, it might be more appropriate to evaluate ALI according to whether the tumour invades an adjacent lobe across the fissure (ALI-A) or directly extends into an adjacent lobe beyond an incomplete fissure (ALI-D). We first performed the prognostic evaluation of NSCLCs with ALI focusing on the interlobar fissure status.

In this study, NSCLC patients with ALI demonstrated different survivals depending on whether their tumours had ALI-A or ALI-D. The outcome of the patients with ALI-D tumours was significantly better than those with ALI-A tumours. The survival of the patients with ALI-A tumours was statistically not different from that of the patients with T2b tumours, regardless of the tumour size. However, the survival of the patients with ALI-D was not statistically different from that of patients with T1 tumours. In spite of a small sample size ($n = 90$) in this study cohort, the type of ALI was a marginally significant prognostic factor in multivariate analysis ($P = 0.097$). The present study suggests that the type of ALI is an important prognostic indicator for ALI tumours and only ALI-A tumours are appropriate to recognized as true ALI tumours. In the 7th TNM staging system, ALI-A tumours with 5 cm or less are appropriate to be assigned to T2b, regardless of the tumour size. On the other hand, ALI-D tumours may not require the adjustment of the T descriptor.

The survival disparity between ALI-A and ALI-D tumours might be explained by the interrelationship between the interlobar visceral pleura and the invading tumour. To be classified as the ALI-A, tumours must be observed to be invading the adjacent lobes through two layers of the visceral pleura, a primary tumour side and an adjacent side. Pleural invasion indicates biological tumour invasiveness and is associated with a poor outcome [1, 2]. From an anatomical point of view, small vascular structures, including lymphatic ducts and blood vessels, are abundant in the subpleural space [12]. ALI-A tumours might have more chances to invade small lymphatic ducts and blood vessels than ALI-D tumours by penetrating two layers of the visceral pleura. Actually, ALI-A tumours had significantly more tumours with vascular invasion than ALI-D tumours in this study (88 vs 61%, $P = 0.009$). Lymphatic permeation was also more frequent in ALI-A tumours than ALI-D tumours, although this was not statistically significant (58 vs 39%, $P = 0.138$). The poor outcome of the patients with ALI-A tumours might be explained by their greater invasiveness into small blood vessels and lymphatic ducts.

In the 7th TNM staging system, the prognostic impact of pleural invasion has been emphasized [9]. Not only the invasion of the pleural surface (PL2), but also the invasion beyond the elastic layer without exposure to the pleural surface (PL1) was regarded as 'VPI' and assigned to T2a. Determination of the extent of pleural invasion using elastic stains is also recommended [2]. There were distinct prognostic differences between ALI-A and ALI-D tumours. Elastic stains enable us to differentiate between the two types of ALIs easily. When we evaluate the tumours suspected to have ALI and that require a combined resection of an adjacent lobe, the interlobar fissure status should be examined in detail by elastic stains.

The tumours with ALI need to be resected by a combined resection of the adjacent lobe. We chose a wedge resection or segmentectomy of the adjacent lobe if a secured surgical margin more than 1 cm was obtained. There was no recurrence in the cut end of the lung parenchyma in the adjacent lobe in our study population. Several researchers have shown equal postoperative survival after a resection of the tumour with ALI between a lobectomy with partial resection and a bilobectomy or pneumonectomy [5–7]. These indicate that a combined partial resection of the adjacent lobe with an adequate surgical margin is a curative resection for the tumours with ALI.

LIMITATIONS

The study's limitations include the small sample size, in particular, the small population with ALI-D tumours. Further validation studies should be conducted in a large cohort to clarify the prognostic impact of the interlobar fissure status on the prognosis of ALI tumours. We hope that our proposal will be validated in a large sample collected by multiple institutions in cooperation with the International Association for the Study of Lung Cancer, and it should be taken into consideration in the next revision of the TNM staging system.

CONCLUSIONS

In conclusion, there were distinct survival differences between the patients with ALI-A and ALI-D tumours according to the interlobar fissure status. ALI should be examined accurately by using elastic stains and only ALI-A should be classified as true ALI. We propose that ALI-A tumours with a size of ≤5 cm should be assigned to T2b. Otherwise, ALI-D tumours do not require the adjustment of the T descriptor.

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Conflict of interest: none declared.

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EDITORIAL COMMENT

Recent advances in our understanding of lung cancer visceral pleural invasion and other forms of minimal invasion: implications for the next TNM classification

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Keywords: Non-small-cell lung cancer • Visceral pleural invasion • Interlobar fissure • Adjacent lobe invasion • Hilar fat invasion

Involvement of the visceral pleura (VP) has long been recognized as an adverse prognostic factor in non-small-cell lung carcinoma (NSCLC). It was shown more recently with the aid of elastic stains that a tumour invading beyond the VP elastic layer has the same prognostic impact as a tumour extending to the VP surface [1]. Based on this and other data, the 7th edition TNM adopted a proposal to classify VP invasion (VPI) as PL0 if the tumour does not invade past the elastic layer, as PL1 if it invades past the elastic layer, PL2 if it invades to the pleural surface and PL3 if it invades to the parietal pleura with the recommendation to classify PL0 as T1, PL1 and PL2 as T2 and PL3 as T3 [1]. However, relatively little progress has been made towards understanding

Prognostic Impact of CD204-Positive Macrophages in Lung Squamous Cell Carcinoma

Possible Contribution of Cd204-Positive Macrophages to the Tumor-Promoting Microenvironment

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Introduction: Tumor-associated macrophages (TAMs) are recruited into cancer-induced stroma and produce a specific microenvironment for cancer progression. CD204 (+) TAMs are reportedly related to tumor progression and clinical outcome in some tumors. The aim of this study was to clarify the correlation between CD204 (+) TAMs and the clinicopathological features of lung squamous cell carcinoma.

Methods: We investigated the relationships between the numbers of CD204 (+) TAMs and clinicopathological factors, microvessel density, and the numbers of Foxp3 (+) lymphocytes in 208 consecutively resected cases. We also examined the relationships between the numbers of CD204 (+) TAMs and the expression levels of cytokines involved in the migration and differentiation of CD204 (+) TAMs.

Results: A high number of CD204 (+) TAMs in the stroma was significantly correlated with an advanced p-stage, T factor, N factor, and the presence of vascular and pleural invasion. A high number of CD204 (+) TAMs in the stroma was also a significant prognostic factor for all p-stages and p-stage I. Moreover, the numbers of CD204 (+) TAMs were correlated with the microvessel density and the numbers of Foxp3 (+) lymphocytes. A high number of CD204 (+) TAMs was strongly correlated with the tissue expression level of monocyte chemoattractant protein-1. CD204 (+) TAMs were shown to be significant independent prognostic factors in a multivariate analysis.

Conclusions: CD204 (+) TAMs were an independent prognostic factor in lung squamous cell carcinoma. CD204 (+) TAMs, along with other tumor-promoting stromal cells such as regulatory T cells and endothelial cells, may create tumor-promoting microenvironments.

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The tumor microenvironment is composed of not only tumor cells, but also stromal cells including macrophages, lymphocytes, fibroblasts, and endothelial cells. Stromal cells are known to interact with cancer cells and to produce a specific microenvironment capable of influencing tumor progression.[1] In the host immune system in cancer tissue, tumor-associated macrophages (TAMs) as well as cancer-associated fibroblasts are important components of the tumor microenvironment, and some kinds of macrophages are known to act on tumor progression.[2] Recent immunological studies have identified two different functions of polarized macrophage activation, as exhibited by classically activated (M1) and alternatively activated (M2) macrophage phenotypes.[3,4] These subpopulations of macrophages have different receptor expression patterns and different cytokine and chemokine productions. M1-polarized macrophages have an interleukin (IL)-12^{high}, IL-23^{high}, IL-10^{low} phenotype and defend the body against pathogens and tumor cells by inducing interferon-gamma and producing tumor necrosis factor α and nitric oxide. M1-polarized macrophages reportedly play a role in tumor suppression. However, M2-polarized macrophages have an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype and have high expression levels of class A scavenger receptor (CD204) and mannose receptor (CD163).[5–7] M2-polarized macrophages play a role in tumor-supportive functions, such as tumorigenesis, angiogenesis, matrix remodeling, and immune-suppression.[8] Recent studies have reported that the number of CD204-positive TAMs within a primary tumor is related to tumor progression and outcome in glioma, ovarian epithelial tumors, pancreatic cancer, and lung adenocarcinoma.[6,9–11]

Although squamous cell carcinoma of the lung is second only to adenocarcinoma of the lung, its treatmentt has not yet been sufficiently effective. Recently, the development of

cancer therapy targeting cancer stromal cells has been proposed.[12] In the current study, we examined whether the numbers of CD204 (+) TAMs recruited into the cancer tissue are related to clinicopathological factors of lung squamous cell carcinoma. Furthermore, we examined the matching correlations between CD204 (+) TAMs and other types of cancer stromal cells, including regulatory T cells and endothelial cells.

MATERIALS AND METHODS

Patients

Between January 2000 and December 2006, a total of 255 patients with lung squamous cell carcinoma underwent surgery with curative intent at our hospital. We excluded 47 cases with poor-quality surgical specimens, and the remaining 208 cases were included in this study. The median follow-up period was 5.7 years.

Histopathology Studies

Surgical specimens were fixed in 10% formalin or methanol and embedded in paraffin. Sections that are 4-μm–thick were stained using the hematoxylin and eosin method. Vascular invasion and pleural invasion were evaluated using the Verhoeff-van-Gieson method. The histologic diagnoses were based on the third revised World Health Organization histologic classification. Disease stages were based on the 7th edition of TNM classification.

Evaluation of Clinicopathological Factors

The clinical characteristics were retrieved from the available clinical records. The following clinicopathological factors were investigated retrospectively to assess their impact on survival: age, sex, smoking history, pathologic stage, pathologic T status, pathologic nodal involvement, lymphatic permeation, vascular invasion, and pleural invasion.

Antibodies and Immunohistochemistry

The slides were deparaffinized with xylene, rehydrated, and antigen-retrieved in a microwave oven for 20 minutes. After the inhibition of endogenous peroxidase activity, individual slides were then incubated overnight at 4°C with mouse antihuman CD204 antibody (Scavenger Receptor class A-E5; Transgenic, Japan) at a final dilution of 1:400, mouse antihuman CD34 antibody (QBEND/10; Acris Antibodies, Herford, Germany) at a final dilution of 1:400, and mouse monoclonal antihuman Foxp3 antibody (236A/E7; Abcam, Japan) at a final dilution of 1:150. The slides were then incubated with EnVision (Dako, Denmark), and the color reaction was developed in 2% 3, 3-diaminobenzidine in 50 mM Tris-buffer (pH7.6) containing 0.3% hydrogen peroxidase. Finally, the sections were counterstained with Meyer hematoxylin.

Evaluation of Immunohistochemistry

Two pathologists (S.H. and G.I.) selected a hot spot within a section and counted the CD204 (+) TAMs in the cancer stroma and nest in five high-power microscopic fields (×400; 0.0625 mm^2). The average counts were recorded and cases were divided into two groups with low and high numbers of CD204 (+) TAMs according to the median value. The absolute number of Foxp3-positive lymphocytes in the stroma, was counted in five randomly selected high-power microscopic fields (×400; 0.0625 mm^2), and the average counts were recorded. As for the microvessel density (MVD), the five most vascular areas (hot spots) in the invasive foci within a section were selected, and vessels labeled with anti-CD34 monoclonal antibody were counted in five high-power microscopic fields (×400; 0.0625 mm^2). The average counts were recorded as the MVD. In these studies, we selected areas in the central area within a cancer tissue, and necrotic areas were excluded.

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

Total RNA was extracted from 13 lung squamous cell carcinoma cases.. Samples of both cancer tissue and noncancerous tissue were collected and immediately homogenized in QIAzol Lysis reagent (QIAGEN, CA) with Tissue Lyser II (QIAGEN) and stored at −80°C until use. The total RNA was isolated from the tissues using a QIAshredderTM (250) (QIAGEN) and an RNeasy Mini Kit (250) (QIAGEN) according to the manufacturer's instructions. The RNA was reverse transcribed to synthesize cDNA using a primerscript RT reagent kit (Takara Biochemicals, Osaka, Japan). To quantitatively compare the mRNA levels of each cytokine, we performed a real-time polymerase chain reaction using SYBR Premix Ex TaqII (Takara) with the Smart Cycler II (Takara). The sense and antisense primers used for quantitative amplification of the cytokine mRNAs and for amplification of glyceraldehyde-3-phosphate dehydrogenase as an internal control are shown in (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A347). The amount of template cDNA was expressed by the threshold cycles (G), determined from the amplification curve (exponential curve), and the threshold level for the detection of the polymerase chain reaction product. The expression level of each gene was reported as the ratio of its expression to the level of glyceraldehyde-3-phosphate dehydrogenase gene expression in the same sample. The ratio between the level of cytokine expression in the cancer tissue to the level of expression in the noncancerous tissue was calculated for each case. The median number of CD204 (+) TAMs was used to divide the cases into high and low CD204 (+) TAM groups.

Statistical Analysis

The distributions of CD204 (+) TAMs in the stroma and MVD and Foxp3-positive lymphocytes were tested for correlations by calculating the Spearman rank correlation coefficients. Overall survival (OS) was measured from the date of surgery until the date of death from any cause or to the date on which the patient was last known to be alive. Recurrence-free survival (RFS) was measured from the date of surgery until the date of recurrence or until the date the patient was last known to be disease free. Survival curves were estimated using the Kaplan–Meier method, and differences in survival

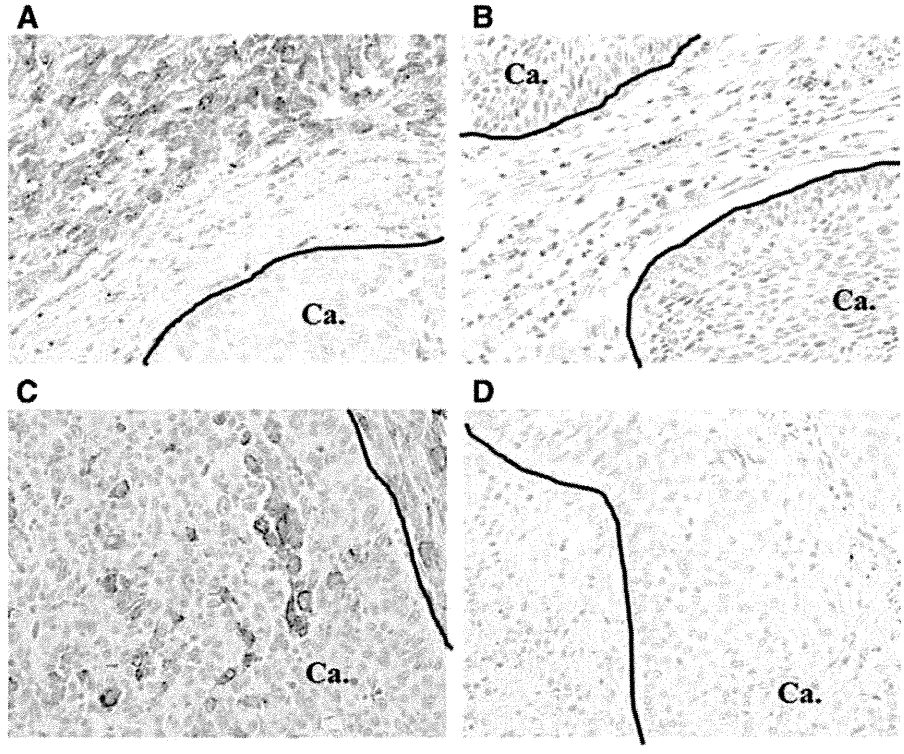

FIGURE 1. *A,* Immunohistochemical staining of squamous cell carcinoma with antihuman CD204 antibody for tumor-associated macrophages in the stroma and nest. Cases with a high number of CD204 (+) TAMs in the stroma, and *B,* a low number of CD204 (+) TAMs in the stroma. *C,* Cases with a high number of CD204 (+) TAMs in the nest, and *D,* a low number of CD204 (+) TAMs in the nest. TAMs, tumor-associated macrophages.

were compared using the log-rank test. A p value less than 0.05 was considered significant. Statistical analysis software (SPSS, version II) was used to perform the analyses.

RESULTS

Correlations between the numbers of CD204 (+) TAMs in the cancer stroma and nest and clinicopathological factors.

A representative case of immunohistochemical staining results for CD204 is shown in Fig. 1. All the patients were classified into two groups according to the median values: 30 for the stroma, and nine for the nest. A high CD204 (+) TAM count in the stroma was significantly correlated with the pathologic stage, pathologic T status, pathologic nodal involvement, and vascular and pleural invasion (Table 1). However, lymphatic permeation was significantly less frequent in the group with a high CD204 (+) TAM count in the nest, compared with those with a low CD204 (+) TAM count in the nest (Supplemental Table 2, Supplemental Digital Content 2, http://links.lww.com/JTO/A348).

Evaluation of CD204 (+) TAMs in Stroma and Nest as Prognostic Factors

The OS and RFS were significantly shorter in the group with a high CD204 (+) TAM count in the stroma compared with the group with a low CD204 (+) TAM count in the stroma, for all stages (p = 0.0005 and p = 0.0002, respectively) (Fig. 2A). In the p-stage I patients, the OS and RFS were also significantly shorter in the group with a high CD204 (+) TAM count in the stroma, compared with the group with a low CD204 (+) TAM count in the stroma (p = 0.0154 and p = 0.0071) (Fig. 2*B*). A high CD204 (+) TAM count in the stroma was marginally related to the OS and RFS among the p-stage II patients (Fig. 2*C*), but no significant relation with prognosis was seen among the p-stage III patients (Fig. 2*D*). In contrast, no relationship was seen between a high CD204 (+) TAM count in the nest and the prognosis (data not shown).

Correlations among the numbers of CD204 (+) TAMs in the stroma, Foxp3-positive lymphocytes, and the microvessel density Recent studies showed that CD204 (+) TAMs contribute to the development of neovascularization and immunesuppression. We examined the numbers of Foxp3 (+) lymphocytes (regulatory T cells) and the MVD in all the cases (Fig. 3A–D). The numbers of CD204 (+) TAMs in the stroma were strongly correlated with the MVD ($p < 0.001$; $r_s = 0.471$) and were moderately correlated with the numbers of Foxp3-positive lymphocytes ($p = 0.034$; $r_s = 0.147$) (Fig. 3*E* and *F*).

Correlations between the numbers of CD204 (+) TAMs in the stroma and cytokine expression in the cancer tissues. We examined the expressions of factors involved in the recruitment of TAMs, regulatory T cells, and endothelial cells. The correlations between MVD monocyte chemoattractant protein-1 (MCP-1), IL-10, transforming growth factor β, and vascular endothelial growth factor (VEGF) expression and the numbers of CD204 (+) TAMs in the tumor tissue specimens (n = 13) were analyzed. The ratios of MCP-1 expression in the cancer tissues to their levels of expression in noncancerous tissues were significantly higher in the CD204 high group (p = 0.032) (Fig. 4A). The ratios of IL-10 and transforming growth factor β expressions in the cancer tissues to their levels of expression in noncancerous tissue were marginal higher in the CD204 high group (p = 0.063 and p = 0.086,

TABLE 1. Relationship between CD204 (+) Tumor-Associated Macrophages in Stroma and Clinical Features

Variables	CD204 Low (n = 93)	CD204 High (n = 115)	p^a
Sex			
Male	85	103	
Female	8	12	0.6556
Age (yr)			
<70	49	63	
70≤	44	52	0.7632
Smoking history			
Never smoker	3	10	
Smoker	90	105	0.1052
Surgical procedures			
Lobectomy + segmentectomy	82	94	
Pneumonectomy	11	21	0.2475
Pathological stage			
Stage I	56	48	
Stage II–IIIA	37	67	0.0081[b]
T states			
T1	34	24	
T2–4	59	91	0.0121[b]
Lymph node metastasis			
N(−)	65	60	
N(+)	28	55	0.0095[b]
Vascular invasion			
V(−)	37	28	
V(+)	56	87	0.0169[b]
Lymphatic permeation			
Ly(−)	71	87	
Ly(+)	22	28	0.9076
Pleural invasion			
P(−)	66	68	
P(+)	27	47	0.018[b]

[a]Pearson 2 test.
[b]p <0.05.

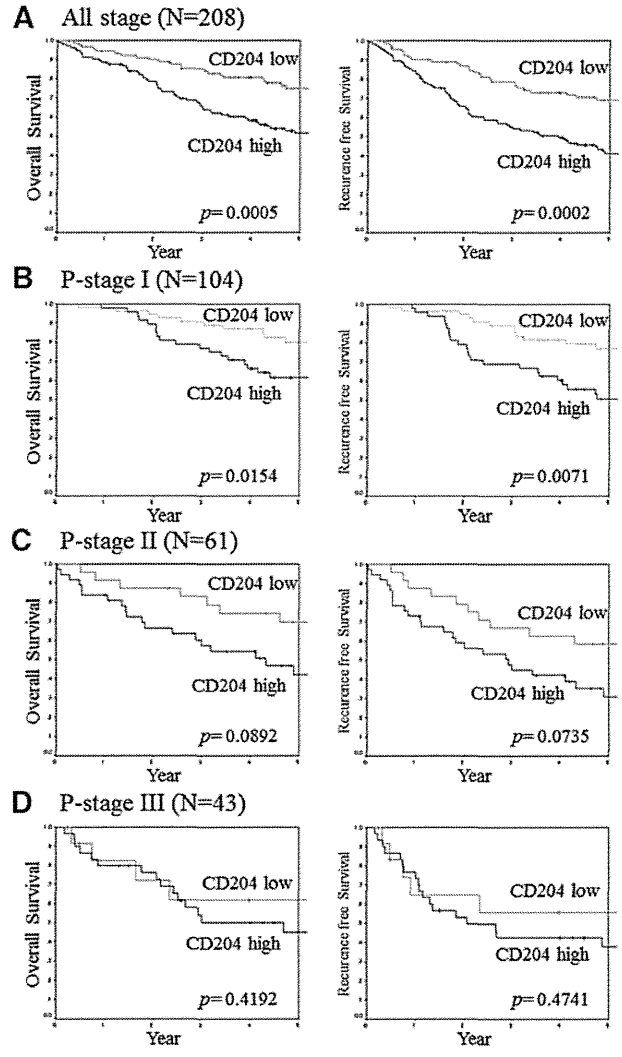

FIGURE 2. Kaplan–Meier analysis stratified according to a high or low number of CD204 (+) TAMs in the stroma. The Kaplan–Meier analysis for overall survival and recurrence-free survival according to the numbers of CD204 (+) TAMs in the stroma are shown for all stages *A*, for p-stage I, *B*, for p-stage II, *C*, and for p-stage III, *D*,. TAMs, tumor-associated macrophages.

respectively) (Fig. 4*B*, *C*). The difference in the expression of VEGF between the two groups was not significant (Fig. 4*D*).

Univariate and Multivariate Analyses of Factors Associated with Prognosis

A univariate analysis identified four significant risk factors for OS: CD204 (+) TAMs in the stroma, p-T status, vessel invasion, and pleural invasion (Table 2). In a multivariate analysis, the presence of CD204 (+) TAMs and the p-T status were shown to be statistically significant independent predictors of the OS (Table 3).

DISCUSSION

In this study, we first showed that the numbers of CD204 (+) TAMs in the tumor stroma, but not in the tumor nest, were correlated with several conventional prognostic factors in squamous cell carcinoma of the lung. Furthermore, we showed that the cases with higher numbers of CD204 (+) TAMs in the stroma had a poor clinical outcome, particularly in the early stages. These results were consisted with previous reports in other types of malignancies, such as glioma, ovarian epithelial tumors, pancreatic cancer, and lung adenocarcinoma.[6,9–11] Moreover, the numbers of CD204 (+) TAMs were related to the numbers of Foxp3 (+) lymphocytes and the MVD. These data suggested that in squamous cell carcinoma tissue, CD204 (+) TAMs, along with other tumor-promoting stromal cells such as regulatory T cells and endothelial cells, create a specific microenvironment that supports tumor progression.

TAMs are known to induce the proliferation, survival, and invasion of tumor cells by producing wide range

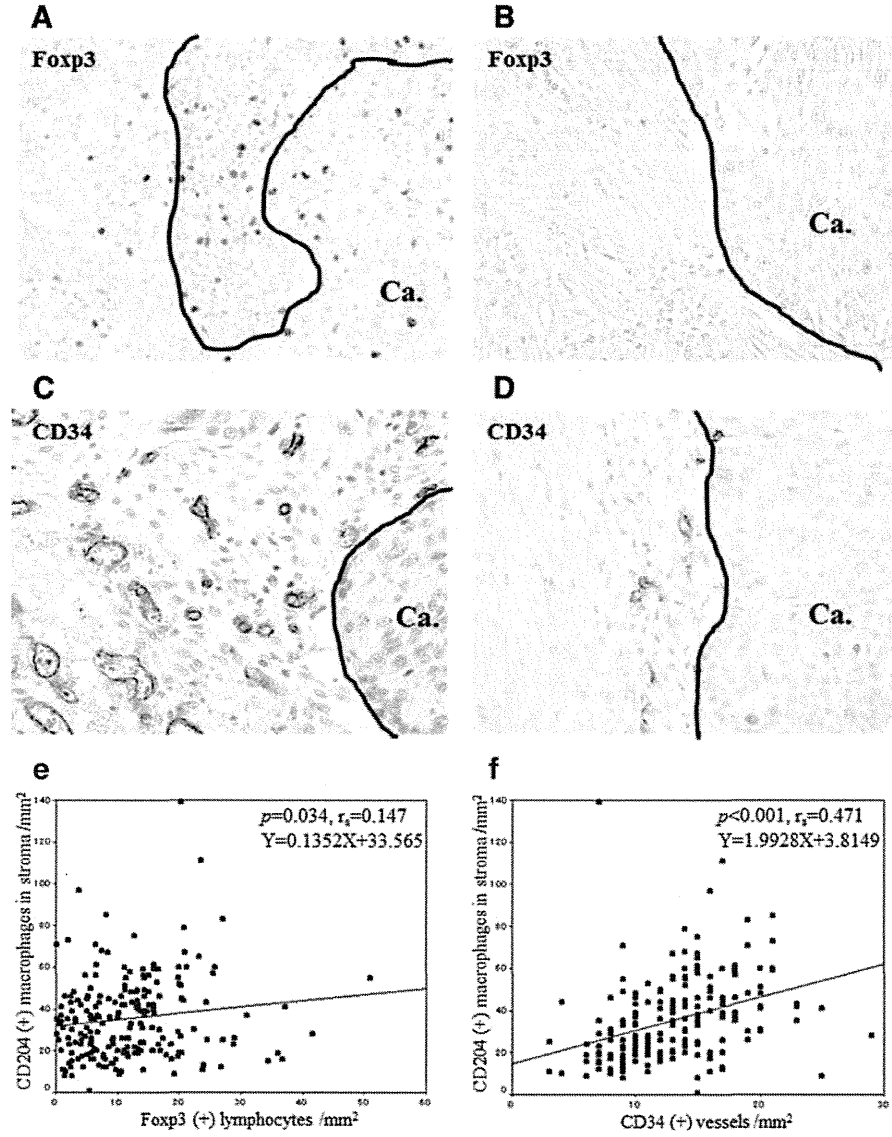

FIGURE 3. Immunohistochemical staining of squamous cell carcinoma tissue with antihuman Foxp3 antibody and antihuman CD34 antibody. *A*, Cases with a high number of Foxp3 positive lymphocytes, and *B*, a low number of Foxp3 positive lymphocytes. *C*, Cases with a high MVD, and *D*, a low MVD. *E*, Correlations between the numbers of CD204 (+) TAMs in the stroma and the MVD, *F*, the number of Foxp3 (+) lymphocytes. TAMs, tumor-associated macrophages; MVD, microvessel density.

of factors, such as matrix metalloproteinases (MMP).[1,13–16] Hagemann et al.[17] reported that TAMs change to M2 phenotype macrophages, CD204 (+) TAMs, after cocultivation with ovarian cancer cells. CD204 (+) TAMs showed a significant up-regulation of mRNA for the genes MMP−1, −2, −7, −9, and −14. Another article reported that the co-cultivation of breast cancer cells with macrophages led to the enhanced invasiveness of the cancer cells as a result of tumor necrosis factor α-dependent MMP-9 secretion from the TAMs.[18] These observations may explain the enhanced vascular and pleural invasion of squamous cell carcinoma cells in the high CD204 (+) TAMs groups in the current study.

We found that the numbers of CD204 (+) TAMs were strongly correlated with the MVD. Kawahara et al.[19] showed similar results that M2 macrophages were correlated with the MVD in gastric cancer. Given the previous report that CD204 (+) TAMs secrete proangiogenic factors, including VEGF,[17] the positive relation observed between CD204 (+) TAMs and the MVD in the present study is understandable. However, the numbers of CD204 (+) TAMs were not associated with the expression of VEGF in the tumor tissue. This discrepancy might be caused by the fact that angiogenesis also depends on angiogenic factors other than VEGF.

The number of CD204 (+) TAMs in the stroma was marginally correlated with the number of Foxp3 (+) lymphocytes, which was partly consistent with the results for intrahepatic cholangiocarcinoma.[20] Foxp3 (+) T cells down-regulate the immune response by attenuating the host's antitumor T cells, potentially permitting unrestricted growth, subsequent metastasis, and recurrence.[21,22] Taking these into consideration, CD204 (+) TAMs may not only enhance tumor cell invasiveness directly, but may also create a more tumor-promoting microenvironment by recruiting endothelial cells and regulatory T cells.

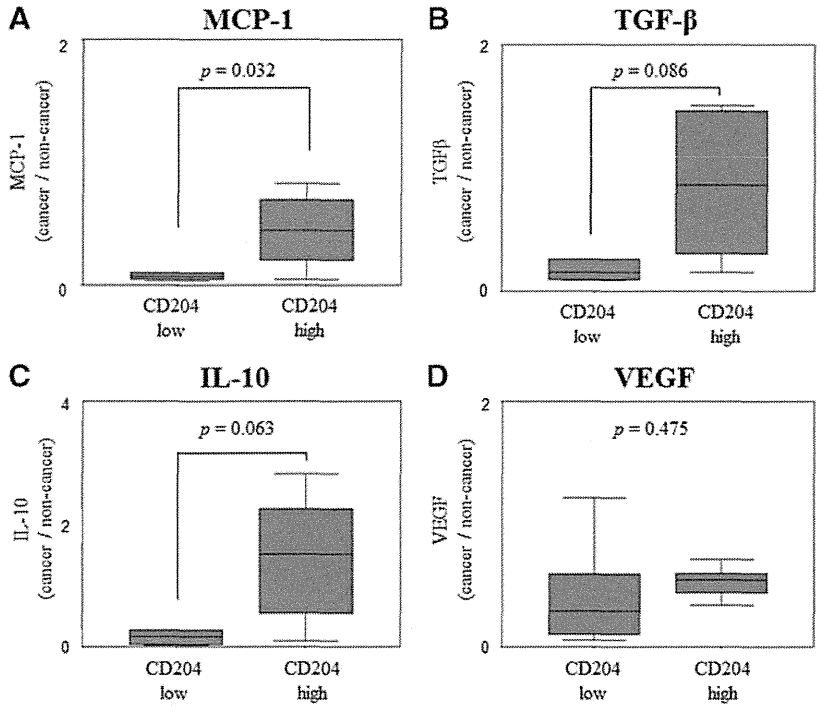

FIGURE 4. Relative mRNA expression in CD204-low and CD204-high cases. The levels of mRNA expression shown are the ratios of expression in cancer tissues relative to the expression in noncancerous tissues, as determined using a quantitative real-time polymerase chain reaction. MCP-1, monocyte chemoattractant protein-1; TGF β, transforming growth factor β; VEGF, vascular endothelial growth factor; IL-10, interleukins-10.

TABLE 2. Univariate Analysis for Overall Survival (N =208)

Variables	Category	All Cases	5-Year Survival (%)	p^a
Age				
Median, yrs (range)	69 (46–88)			0.1436
	<70	112	66	
	≥70	96	58	
Sex	Female	20	48	0.3689
	Male	188	63	
Smoking	Never	13	35	0.1894
	Ever	195	64	
Surgical procedures	Lobectomy segmentectomy	176	63	0.2908
	Pneumonectomy	32	56	
T factor	≤T1	58	84	0.0006[b]
	>T1	150	54	
N factor	pN0	125	66	0.1891
	pN1/pN2	83	56	
Lymphatic permeation	Absent	158	61	0.4973
	Present	50	67	
Vascular invasion	Absent	65	73	0.0381[b]
	Present	143	57	
Pleural invasion	Absent	134	70	0.0013[b]
	Present	74	47	
CD204 (+) TAMs in stroma	Low	93	75	0.0005[b]
	High	115	52	
CD204 (+) TAMs in nest	Low	91	63	0.5894
	High	117	61	

[a]Log-rank test.
[b]p 0.05.
TAMs, tumor-associated macrophages.

TABLE 3. Multivariate Analysis for Overall survival

Variables	Favorable	Unfavorable	Overall Survival		
			Hazard Ratio	95% Confidence Interval	*p*
T factor	≤T1	>T1	0.486	0.246–0.957	0.037
Vascular invasion	Absent	Present	1.124	0.650–1.942	0.676
Pleural invasion	Absent	Present	1.447	0.895–2.340	0.132
CD204 (+) TAMs in stroma	Low	High	2.053	1.273–3.311	0.003

TAMs, tumor-associated macrophages.

In the present study, we showed that the tissue expression of MCP-1 was significantly correlated with the numbers of CD204 (+) TAMs. MCP-1 has been reported as a key cytokine that induces the migration, accumulation, and differentiation of the M2 phenotype and contributes to the recruitment of CD204 (+) TAMs into the tumor tissue.[11,23] Moreover, MCP-1 can act directly on endothelial cells to promote angiogenesis.[24] Although no significant association was seen between the number of CD204 (+) TAMs and the VEGF mRNA level, MCP-1 might contribute to an increase in the MVD.

In this study, there 10 patients received postoperative adjuvant chemotherapy and 21 patients received chemotherapy after recurrence. However, there are no differences in the prognosis with or without postoperative adjuvant chemotherapy (RFS; $p = 0.2329$, OS; $p = 0.2548$) and chemotherapy after recurrence (OS; $p = 0.1318$). Among 198 patients who did not receive adjuvant chemotherapy, a high number of CD204 (+) TAMs in the stroma was also a significant prognostic factor for all *p*-stages and *p*-stage I (All p-stages: RFS $p = 0.0002$, OS $p = 0.0012$, *p*-stageI: RFS $p = 0.0169$, OS $p = 0.0369$). Therefore CD204-positive TAM was a strongly independent prognostic factor, even subtracting the effect of treatment.

In a recent report, the actions of bisphosphonates on macrophages not only impaired TAMs recruitment, but also inhibited the release of proangiogenic factors capable of affecting TAMs by reversing their polarization from the M2 to the M1 phenotype.[25] Moreover, the depletion of TAMs by clodrolip, which consists of a liposome encapsulating clodronate or zoledronic acid in combination with sorafenib, significantly inhibited tumor progression in hepatocellular carcinoma in vitro.[26] Our current results suggest that the targeting of CD204 (+) TAMs may be useful as a supplemental therapy for conventional cancer-treatment regimens for lung squamous cell carcinoma.

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