Organization's histological classification and were staged according to the TNM classification of the seventh edition of the Union for International Cancer Control. Furthermore, per the WHO classification, we organized all the cases into the four subtypes focused on their histological predominance: bronchioloalveolar carcinoma, acinar, papillary, or solid adenocarcinoma with mucin production.

Antibodies and immunohistochemistry. After the pathologic assessment of the H&E-stained slides of the surgical specimens, 4-μm thick sections were made from formalin-fixed, paraffin-embedded specimens. Each slide was then incubated overnight at 4°C with mouse antihuman Foxp3 antibodies (ab20034 clone 236A/E7 diluted at 1:200; Abcam Inc, Cambridge, MA, USA). The slides were incubated with EnVision™ (Dako, Glostrup, Denmark) for 30 min at room temperature for visualization of bound primary antibody. They were visualized in 2% 3,3′-diaminobenzidine in 50-mM Tris buffer (pH 7.6) containing 0.3% hydrogen peroxidase.

Immunohistochemical scoring. All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (T.K. and G.I.) who did not know any clinicopathological information regarding the cases. Among tumor-infiltrating lymphocytes, T_{reg} were detected based on the presence of positive nuclear staining for Foxp3. The absolute numbers of T_{reg} in the stroma and in the nest were counted in five different high-power fields (HPF, ×400 magnification). We counted T_{reg} in the clear hot spot, and average cell count was defined as the T_{reg} number.

Statistical analysis. Overall survival (OS) was measured from the date of surgery until the date of death from any cause or the date on which the patient was last known to be alive. The recurrence-free survival (RFS) time was measured as the interval 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 disease-free. Survival curves were plotted according to the Kaplan–Meier method and were compared using the log-rank test. Two-category comparisons were performed using the Pearson chi-squared test or the Mann–Whitney *U*-test for quantitative data. All the tests were two sided, and *P*-values <0.05 were considered statistically significant. The statistical analysis was performed using StatView version 5.0 for Windows (SAS Institute Inc., Cary, NC, USA).

Fibroblast culture. Both CAF and non-cancer-associated fibroblasts were prepared from human lung cancer tissue and non-cancerous lung tissue obtained from the same specimen as previously reported.[15,16] Briefly, an approximately 5-mm^3 sample of carcinoma from each tissue specimen was cut into about 15 subdivisions and placed in an minimum essential medium alpha modification (α-MEM; Sigma, St. Louis, MO, USA) culture containing 10% heat-inactivated FBS and antibiotics (penicillin and streptomycin). The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. After 10–20 days of growth, the fibroblasts were separated from the epithelial and endothelial cells using differential trypsinization. When the cells reached 80% confluence, they were harvested with 0.25% trypsin and 1-mmol/L ethylene-diamine-tetra-acetic acid and replated at a density of 1×10^4 cells/cm^2.

CD4+CD25− cell purification and induction of Foxp3. For induction of CD4+Foxp3+ Treg cells, CD4+CD25−CD45RA+ naive conventional T cells were purified from the peripheral blood cells obtained from two healthy volunteers with a naive CD4+ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, 5×10^5 naive T cells were cultured in the culture supernatant from CAF with 4×10^7 beads/mL CD3/CD28 beads-T cell expander (Invitrogen, Carlsbad, CA, USA) and 10-U/μL recombinant human interleukin (hIL)-2 (Roche, Penzberg, Germany) for 144 h at 37°C in a humidified atmosphere of 5% CO_2. For the control experiments, naive CD4+ T cells were cultured in RPMI1640 containing 10% FBS and 1% penicillin/streptomycin/glutamine with 50-ng/μL recombinant human transforming growth factor-β1 (Peprotech, Rocky Hill, NJ, USA), CD3/CD28 beads-T cell expander, and recombinant hIL-2. The fresh medium was replaced every other day. After 6 days of culture, living T cells were collected from them.

Real-time RT-PCR. The cultured T cells, CAF, and non-cancer-associated fibroblasts were washed with PBS, suspended in 1 mL TRIzol (Invitrogen), and then stored at −80°C. Total RNA was purified from the thawed samples with standard techniques, and cDNA was synthesized with the PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. Real-time RT-PCR was performed using a Smart Cycler System (TaKaRa) and SYBR Premix Ex Taq (TaKaRa), according to the manufacturer's instructions. Next, Foxp3 mRNA expression in the incubated T cells was analyzed with RT-PCR. We compared each Foxp3 expression level in proportion to the total cell number. To normalize the mRNA expression of cytokines such as TGF-β, VEGF, interleukin-10, and COX-2, we calculated the expression ratio (CAF/non-cancer-associated fibroblasts) and defined this ratio relative to the cytokine expression in CAF.

Results

Immunohistochemical staining. We detected T_{reg} among the tumor-infiltrating lymphocytes in the tumor stroma and the tumor nest based on the presence of positive nuclear staining for Foxp3. The average number of T_{reg} ranged from 0.0 to 23.0 (mean: 6.0, median: 5.5) per HPF in the stroma. The typical staining results for T_{reg} in the stroma are shown in Figure 1(a,b). In contrast, the average number of T_{reg} in the nest ranged from 0.0 to 6.4 (mean: 0.4, median: 0) per HPF. The typical staining results for T_{reg} in the nest are shown in Figure 1(c,d). Clearly, the absolute number of T_{reg} was much higher in the stroma than in the nest ($P < 0.01$) (Fig. 1e).

Correlations between number of T_{reg} and clinicopathological features. The study cohort included 88 men and 112 women, with a median of age of 65 years (range: 20–84 years, SD: 9.5 years). Five (2.5%) underwent a pneumonectomy and 195 (97.5%) underwent a lobectomy. The follow-up periods ranged from 3 to 87 months (median follow-up for surviving patients: 73 months).

We examined the clinicopathological characteristics of the cases according to the T_{reg} number in the tumor stroma and tumor nest. To assess the correlation between the clinicopathological characteristics and the T_{reg} number, we divided the patients into two groups according to their mean T_{reg} count (6/HPF). The group in which the T_{reg} count in the stroma was lower than 6/HPF ($n = 107$) was the low T_{reg} group, and the group with a T_{reg} count of 6/HPF or higher ($n = 93$) was the high T_{reg} group. The mean number of T_{reg} in the nest was 0.4/HPF, which was much lower than that in the stroma. The group in which the T_{reg} count in the nest was lower than 0.4/HPF ($n = 124$) was the low T_{reg} group, and the group with a T_{reg} count of 0.4/HPF or higher ($n = 76$) was the high T_{reg} group. For the stroma data, a large tumor diameter ($P = 0.04$), a high serum carcinoembryonic antigen level ($P = 0.03$), the presence of vascular invasion ($P < 0.01$), and the presence of pleural invasion ($P < 0.01$) were significantly more common among the high T_{reg} group than among the low T_{reg} group. For the nest data, a significant difference in vascular invasion ($P = 0.03$) was noted between the two groups, but no apparent differences in the tumor diameter, lymphatic permeation, or pleural invasion were seen (Table 1).

Comparison of T_{reg} number in the stroma according to predominant histological subtypes. To examine the difference in the T_{reg} number according to the predominant histologic subtype,

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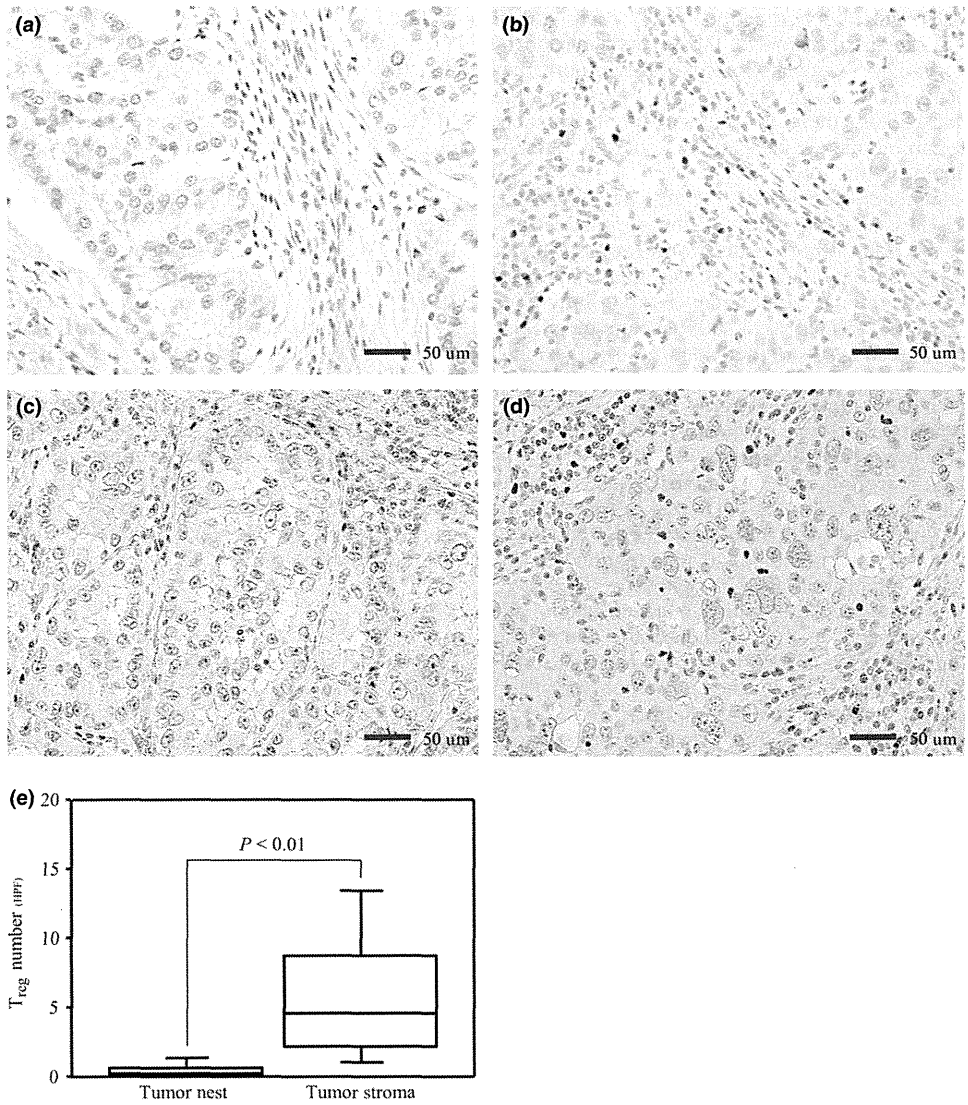

Fig. 1. Representative immunohistochemical findings for Forkhead box P3 (Foxp3+) expression in lymphocytes: (a) low T_{reg} in the stroma, (b) high T_{reg} in the stroma, (c) low T_{reg} in the nest, and (d) high T_{reg} in the nest. (e) Comparison of T_{reg} counts in the nest and the stroma. All analyses were performed using the Mann–Whitney *U*-test. T_{reg}, regulatory T cells.

we divided the cases into four groups: bronchioloalveolar carcinoma, papillary, acinar, and solid (Fig. 2). A difference in the T_{reg} number between the predominantly papillary tumors (n = 83, range: 0.2–27.4, mean: 6.7, median: 5.4) and the predominantly acinar tumors (n = 14, range: 1.0–14.4, mean: 6.9, median: 6.4) was not apparent (P = 0.79). However, the T_{reg} number in the predominantly bronchioloalveolar carcinoma tumors (n = 79, range: 0.2–22.9, mean: 4.0, median: 2.6) was much lower than that in the other histological types (P < 0.01), and that in the predominantly solid tumors (n = 24, range: 3.6–22.4, mean: 11.8, median: 11.6) was significantly higher than those in the other groups (P = 0.01). In the tumor nest, these differences could not be observed (data not shown).

Survival analysis according to number of T_{reg} in the tumor stroma. Figure 3(a) shows the OS curves according to the results of the T_{reg} count (high T_{reg} *vs* low T_{reg}) in the tumor stroma. The 5-year OS rates of the high T_{reg} and low T_{reg} groups were 85.4% and 93.0%, respectively. The OS of the high T_{reg} group was significantly shorter than that of the low T_{reg} group (P = 0.03). Also, RFS curves were plotted according to the results of the T_{reg} count (Fig. 3b). The 5-year RFS rates of the high T_{reg} and low T_{reg} groups were 76.5% and 87.0%, respectively. The RFS of the high T_{reg} group was significantly shorter than that of the low T_{reg} group (P = 0.02).

Induction of T_{reg} from naive CD4+ T cells by CAF. Because we found that the tumor stroma, which is mainly composed of CAF, was the main location of the T_{reg}, we investigated whether soluble factors secreted by CAF influenced the induction of T_{reg}. We selected CAF from 12 cases: six from low T_{reg} adenocarcinomas and six from high T_{reg} adenocarcinomas (Fig. 4a). Next, we cultured the naive T cells according to the above-described method using supernatant samples from these CAF. The results are shown in Figure 4(b). Compared with the low T_{reg} CAF, a significantly higher number of Foxp3+ T cells were induced by the supernatant from the high T_{reg} CAF (P = 0.01).

Correlations between number of T_{reg} and mRNA levels of immunoregulatory cytokines in CAF. We examined whether a correlation was present between the T_{reg} number in the tumor

Table 1. Correlation between T_{reg} number and clinicopathological factors

	Stroma			Nest		
	Low T_{reg} n = 107 (%)	High T_{reg} n = 93 (%)	P-value	Low T_{reg} n = 124 (%)	High T_{reg} n = 76 (%)	P-value
Sex						
Men	48 (44.9)	40 (43.0)	0.89	54 (43.5)	34 (44.7)	0.88
Women	59 (55.1)	53 (57.0)		70 (56.5)	42 (55.3)	
Age (year)						
<65	51 (47.7)	55 (59.1)	0.12	67 (54.0)	39 (51.3)	0.77
≥65	56 (52.3)	38 (40.9)		57 (46.0)	37 (48.7)	
Smoking status						
Never	50 (46.7)	47 (50.5)	0.67	60 (48.4)	36 (47.4)	>0.99
Ever	57 (53.3)	46 (49.5)		64 (51.6)	40 (52.6)	
Tumor diameter (cm)						
≤3	84 (78.5)	60 (64.5)	0.04	90 (72.6)	54 (71.1)	0.87
>3	23 (21.5)	33 (35.5)		34 (27.4)	22 (28.9)	
Serum CEA (ng/mL)						
<5	83 (77.6)	59 (63.4)	0.03	95 (76.6)	47 (61.8)	0.07
≥5	24 (22.4)	34 (36.6)		29 (23.4)	29 (38.2)	
Lymphatic permeation						
Negative	101 (94.4)	80 (86.0)	0.05	114 (91.9)	67 (88.2)	0.45
Positive	6 (5.6)	13 (14.0)		10 (8.1)	9 (11.8)	
Vascular invasion						
Negative	93 (86.9)	59 (63.4)	<0.01	101 (81.5)	51 (67.1)	0.03
Positive	14 (13.1)	34 (36.6)		23 (18.5)	25 (32.9)	
Pleural invasion						
Negative	99 (92.5)	69 (74.2)	<0.01	109 (87.9)	59 (77.6)	0.07
Positive	8 (7.5)	24 (25.8)		15 (12.1)	17 (22.4)	

Two-category comparison was performed using the Pearson's χ^2. CEA; carcinoembryonic antigen; T_{reg}, regulatory T cells.

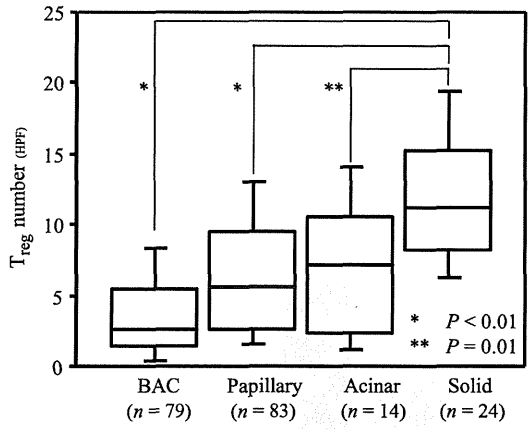

Fig. 2. Comparison of T_{reg} number in the stroma between predominant histological subtypes. All analyses were performed using the Mann–Whitney U-test. BAC, bronchioloalveolar carcinoma; T_{reg}, regulatory T cells.

stroma and the CAF-induced expression levels of immunoregulatory cytokines. RNA samples from cultured fibroblasts isolated from lung cancer tissue (CAF) in 12 adenocarcinoma patients were analyzed by RT-PCR. The results are shown in Figure 5. The relative TGF-β expression levels (median ± SD) induced by the CAF were 1.8 ± 0.4 in the high T_{reg} group and 0.9 ± 0.5 in the low T_{reg} group. In the same way, the relative VEGF expression levels induced by the CAF were 2.6 ± 1.1 in the high T_{reg} group and 1.0 ± 0.4 in the low T_{reg} group. The relative interleukin-10 expression levels induced by the CAF were 1.9 ± 3.2 in the high T_{reg} group and 1.0 ± 1.2 in the low T_{reg} group. The relative COX-2 expression levels induced by the CAF were 1.9 ± 3.2 in the high T_{reg} group and 1.0 ± 1.3 in the low T_{reg} group. No apparent difference in the expression levels of interleukin-10 ($P = 0.52$) or COX-2 ($P = 0.78$) were seen, but significant differences in the TGF-β ($P = 0.01$) and VEGF ($P = 0.01$) levels were detected between the two groups.

Discussion

The accumulation of tumor-infiltrating T_{reg} has been reported to be an unfavorable prognostic marker in several types of carcinomas.[4–8] Petersen *et al.* reported that infiltrating T_{reg} were associated with the recurrence of pathological stage I NSCLC, but they did not mention the influence of T_{reg} on overall survival.[7] We first found that the T_{reg} number in the tumor stroma was a significant indicator of a poor outcome with regard to overall, recurrence-free and disease-specific survival in p-stage I lung adenocarcinoma. Although the T_{reg} number was not an independent prognostic factor in multivariate analysis (data not shown), the presence of T_{reg} in the tumor stroma may encourage an unfavorable prognosis in patients with lung adenocarcinoma.

Growing evidence suggests that T_{reg} play an important role in suppressing T cell-mediated immunity in patients with cancer.[17,18] The number of T_{reg} in the tumor stroma was much larger than in the tumor nest. Commonly, CAF are located in the stroma and are distinctively detected in invasive carcinomas, including NSCLC. Therefore, we focused on the relationship between T_{reg} and the characteristics of CAF. We first hypothesized that cytokines secreted by CAF may have an important role in T_{reg} induction in the stroma, and we examined whether T_{reg} could be induced by soluble factors secreted by CAF derived from high T_{reg} and low T_{reg} adenocarcinomas. Compared with the low T_{reg} CAF, the high T_{reg} CAF induced a

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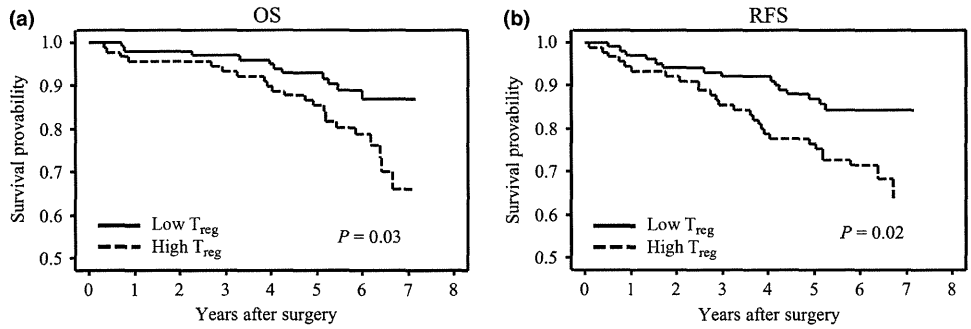

Fig. 3. Kaplan–Meier (a) overall survival curve and (b) recurrence-free survival curve for patients with p-stage I invasive lung adenocarcinoma according to the T_{reg} number in the stroma. The log-rank test was used for all the survival analyses. OS, overall survival; RFS, recurrence free survival; T_{reg}, regulatory T cells.

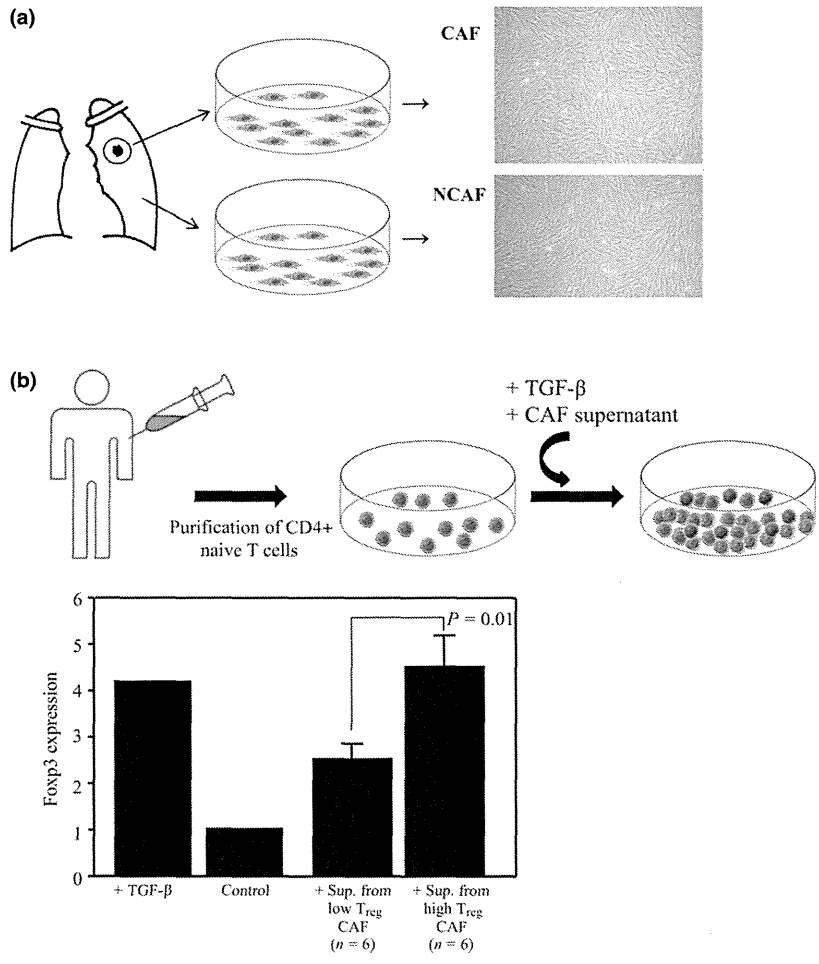

Fig. 4. (a) CAF and NCAF were prepared from human lung cancer tissue and non-cancerous lung tissue obtained from the same samples. After 10 to 20 days of growth, the fibroblasts were cultured as previously reported. (b) Induction of T_{reg} from naive CD4+ T cells by CAF. We selected CAF and cultured the naive T cells according to the above-described method using supernatant samples from 12 cases: six from low T_{reg} adenocarcinomas and six from high T_{reg} adenocarcinomas. CAF, cancer-associated fibroblasts; NCAF, non-cancer-associated fibroblasts; Sup., supernatant; TGF-β, transforming growth factor-β; T_{reg}, regulatory T cells.

significantly larger number of T_{reg} from CD4+ naive T cells. Furthermore, we examined the expression of four kinds of immunoregulatory cytokines from 12 adenocarcinoma cases. The expressions of TGF-β and VEGF, which reportedly induce T_{reg} from naive T cells in the periphery,[19,20] were significantly higher in the CAF from high T_{reg} adenocarcinomas than in the CAF from low T_{reg} adenocarcinomas. CAF are known to produce mainly TGF-β and VEGF, compared with cancer cells.[21,22] We suggest that the CAF in high T_{reg} adenocarcinomas may have a higher immunoregulatory cytokine-secreting capacity, leading to T_{reg} induction. Although we did not evaluate the influence of cytokine expression from tumor cells and tumor-associated macrophages in the present study. Saji *et al.* confirmed that tumor-infiltrating stromal cells were major sources of TGF-β based on the results of an immunohistochemical analysis in NSCLC.[23] Thus, the characteristics of CAF may have a great influence on tumor progression via the recruitment of other types of tumor-promoting stromal cells. There have been no reports demonstrating the correlation between the T_{reg} induction/recruitment and characteristics of CAF. It is

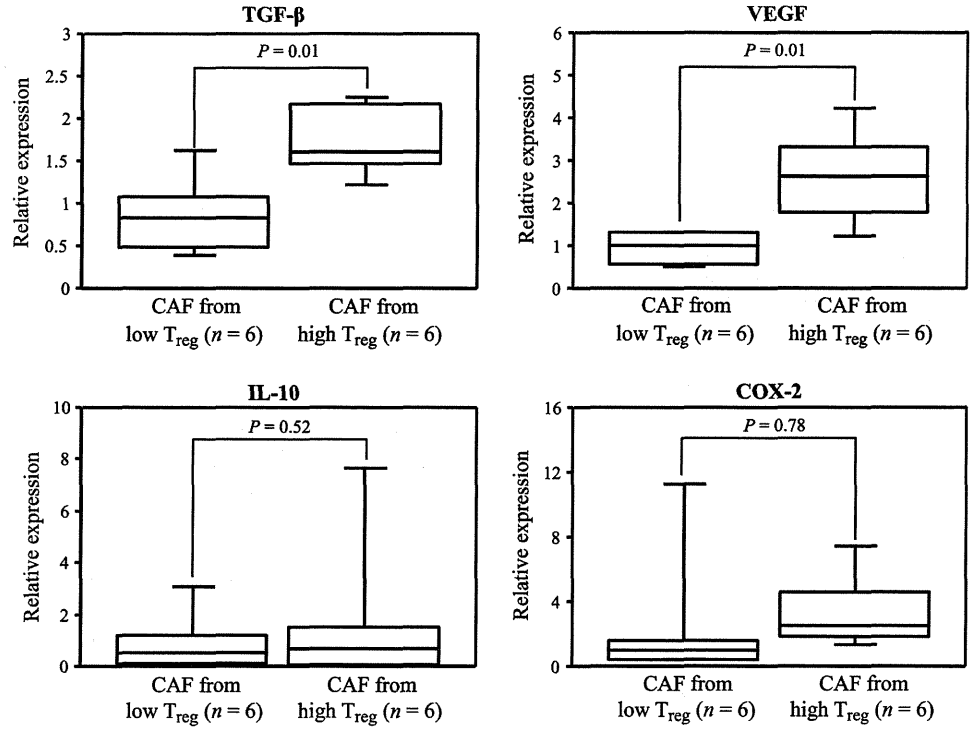

Fig. 5. Relative expression levels of immunoregulatory cytokines expressed by CAF (CAF/NCAF). Total RNA was purified from thawed samples, and cDNA was synthesized. All the analyses were performed using the Student's *t*-test. CAF, cancer-associated fibroblasts; IL-10, interleukin-10; NCAF, non-cancer-associated fibroblasts; TGF-β, transforming growth factor-β; T_{reg}, regulatory T cells; VEGF, vascular endothelial growth factor.

well-known that cytokines, other than TGF-β and VEGF, are also concerned with the T_{reg} induction. Examining the gene expression profile through microchip analysis will be helpful for elucidating which cytokines are upregulated in high T_{reg} CAF.

Recently, Miyao *et al.* reported that there was a minor population of non-regulatory Foxp3+ T cells exhibiting promiscuous and transient Foxp3 expression that were induced from the naive T cells in the peripheral lymph nodes.[24] In order to demonstrate whether these induced Foxp3+ T cells have immunoregulatory function, we would need to divide induced T_{reg} into groups according to CD25 expression levels and compare the immunosuppressive ability thereafter. In the current study, we did not examine the influence of chemokines. In the tumor microenvironment, there are many kinds of stromal cells such as macrophages and monocytes. It is possible these stromal cells educate CAF, which could create some chemokines and the T_{reg}-abundant microenvironment.

In lung cancer, Tao *et al.* reported the influence of T_{reg} in tumor stroma on OS and RFS, but did not mention the correlation between the T_{reg} count and tumor malignant parameters.[6] In the current study, we found that the high T_{reg} group, more frequently than the low T_{reg} group, had predictors of a poor outcome such as a large tumor diameter, vessel invasion, and pleural invasion. These differing results may have occurred because we examined T_{reg} in all stage of NSCLC, including squamous cell carcinomas and large cell carcinomas.

Additionally, the impact of the histological features of lung adenocarcinomas on T_{reg} accumulation has not been previously reported. In this study, the T_{reg} count was highest among patients with a predominantly solid adenocarcinoma subtype. Lung adenocarcinoma patients with a solid adenocarcinoma component are known to have a poorer prognosis than patients without this component.[25] The tumor microenvironment of a solid component probably recruits and induces more T_{reg} than other histological subtypes, enabling both the tumor cells to evade the immune system and the tumor to progress easily. Thus, we suggest that tumor cells acquire the ability to survive and metastasize as a result of a tolerance in antitumor immunity induced by T_{reg} in the tumor stroma as the tumor progresses.

In conclusion, we showed that lung adenocarcinoma with a large number of T_{reg} in the tumor stroma was associated with a poor outcome among patients with p-stage I lung adenocarcinoma after complete resection. Additionally, CAF overexpressing immunoregulatory cytokines, such as TGF-β and VEGF, may play an important role in T_{reg} induction. Recently, numerous reports have described the treatment of patients with colorectal cancer, breast cancer, or lung cancer using humanized monoclonal anti-VEGF antibody therapy (bevacizumab). To confirm the effectiveness of anti-VEGF antibody as an adjuvant therapy in patients with abundant T_{reg} in the tumor stroma, further studies are needed to elucidate the relationship between T_{reg} and CAF. Understanding this relationship will enable the creation of efficacious follow-up plans and improved therapeutic options for patients.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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References

1 Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009; **59**: 225–49.
2 Pisters KM, Evans WK, Azzoli CG *et al.* Cancer Care Ontario and American Society of Clinical Oncology adjuvant chemotherapy and adjuvant radiation therapy for stages I-IIIA resectable non small-cell lung cancer guideline. *J Clin Oncol* 2007; **25**: 5506–18.
3 Johansson M, Denardo DG, Coussens LM. Polarized immune responses differentially regulate cancer development. *Immunol Rev* 2008; **222**: 145–54.
4 Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3$^+$ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006; **12**: 5423–34.
5 Bates GJ, Fox SB, Han C *et al.* Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006; **24**: 5373–80.
6 Tao H, Mimura Y, Aoe K *et al.* Prognostic potential of FOXP3 expression in non-small cell lung cancer cells combined with tumor-infiltrating regulatory T cells. *Lung Cancer* 2012; **75**: 95–101.
7 Petersen RP, Campa MJ, Sperlazza J *et al.* Tumor infiltrating Foxp3$^+$ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 2006; **107**: 2866–72.
8 Shimizu K, Nakata M, Hirami Y, Yukawa T, Maeda A, Tanemoto K. Tumor-infiltrating Foxp3$^+$ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. *J Thorac Oncol* 2010; **5**: 585–90.
9 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat Immunol* 2003; **4**: 330–6.
10 Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 2010; **10**: 490–500.
11 Ishii G, Sangai T, Ito T *et al. In vivo* and *in vitro* characterization of human fibroblasts recruited selectively into human cancer stroma. *Int J Cancer* 2005; **117**: 212–20.
12 Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006; **6**: 392–401.
13 Bremnes RM, Donnem T, Al-Saad S *et al.* The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. *J Thorac Oncol* 2011; **6**: 209–17.
14 Xing F, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front Biosci* 2010; **15**: 166–79.
15 Hoshino A, Ishii G, Ito T *et al.* Podoplanin-positive fibroblasts enhance lung adenocarcinoma tumor formation: podoplanin in fibroblast functions for tumor progression. *Cancer Res* 2011; **71**: 4769–79.
16 Ishii G, Hashimoto H, Asada K *et al.* Fibroblasts associated with cancer cells keep enhanced migration activity after separation from cancer cells: a novel character of tumor educated fibroblasts. *Int J Oncol* 2010; **37**: 317–25.
17 Gajewski TF, Meng Y, Blank C *et al.* Immune resistance orchestrated by the tumor microenvironment. *Immunol Rev* 2006; **213**: 131–45.
18 Beyer M, Schultze JL. Regulatory T cells in cancer. *Blood* 2006; **108**: 804–11.
19 Zhang L, Yi H, Xia XP, Zhao Y. Transforming growth factor-beta: an important role in CD4$^+$CD25$^+$ regulatory T cells and immune tolerance. *Autoimmunity* 2006; **39**: 269–76.
20 Wada J, Suzuki H, Fuchino R *et al.* The contribution of vascular endothelial growth factor to the induction of regulatory T-cells in malignant effusions. *Anticancer Res* 2009; **29**: 881–8.
21 Dong J, Grunstein J, Tejada M *et al.* VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. *EMBO J* 2004; **23**: 2800–10.
22 Nakagawa H, Liyanarachchi S, Davuluri RV *et al.* Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene* 2004; **23**: 7366–77.
23 Saji H, Nakamura H, Awut I *et al.* Significance of expression of TGF-beta in pulmonary metastasis in non-small cell lung cancer tissues. *Ann Thorac Cardiovasc Surg* 2003; **9**: 295–300.
24 Miyao T, Floess S, Setoguchi R *et al.* Plasticity of Foxp3$^+$ T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity* 2012; **36**: 262–75.
25 Ohtaki Y, Yoshida J, Ishii G *et al.* Prognostic significance of a solid component in pulmonary adenocarcinoma. *Ann Thorac Surg* 2011; **91**: 1051–7.

CHEST

Original Research

LUNG CANCER

Podoplanin-Positive Cancer-Associated Fibroblasts Could Have Prognostic Value Independent of Cancer Cell Phenotype in Stage I Lung Squamous Cell Carcinoma

Usefulness of Combining Analysis of Both Cancer Cell Phenotype and Cancer-Associated Fibroblast Phenotype

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Background: **The prognostic significance of the tumor microenvironment, which is created by both cancer cells and cancer-associated fibroblasts (CAFs), has been increasingly recognized. The purpose of this study was to analyze the prognostic markers of stage I squamous cell carcinoma (SqCC), with special reference to the immunophenotypes of both cancer cells and CAFs.**

Methods: **A total of 142 patients with stage I SqCC were included in this study. We examined the expressions of E-cadherin, laminin-5, podoplanin, c-MET, carbonic anhydrase IX (CA-IX), CD10, and CD44 in the cancer cells and those of podoplanin, CA-IX, CD10, and CD44 in the CAFs to evaluate their prognostic value.**

Results: **Patients with low E-cadherin expression in the cancer cells showed a significantly poorer prognosis than those with high E-cadherin expression in the cancer cells ($P < .001$). On the other hand, high podoplanin expression in the CAFs was also associated with a significantly poorer prognosis ($P < .001$). A multivariate analysis identified low E-cadherin expression in the cancer cells and high podoplanin expression in the CAFs as significantly independent prognostic factors for overall survival ($P = .013$ and $P = .0011$, respectively). According to subgroup analyses combining E-cadherin expression in cancer cells and podoplanin expression in CAFs, 5-year overall survival of patients with low E-cadherin expression in the cancer cells and high podoplanin expression in the CAFs was 7.0% and showed a significantly poorer prognosis as compared with other groups ($P < .001$).**

Conclusions: **The current study indicates that immunophenotypes of CAFs could have a prognostic value independent of those of the cancer cells in SqCC.** ***CHEST 2013; 143(4):963–970***

Abbreviations: CAF = cancer-associated fibroblast; CA-IX = carbonic anhydrase IX; NSCLC = non-small cell lung cancer; SqCC = squamous cell carcinoma

Cancer tissue is composed of cancer cells and stromal cells around the cancer cell nests. It has been hypothesized that these stromal cells are functionally organized to influence the proliferation and/or survival of the cancer cells and to generate a favorable microenvironment for cancer cell growth. Accumulating evidence suggests that stromal fibroblasts (cancer-associated fibroblasts [CAFs]) may promote tumor growth by several mechanisms, such as by inducing angiogenesis,[1] recruiting bone marrow-derived cells,[2,3] and inducing remodeling of the extracellular matrix.[4-6] The contribution of CAFs to the development of a variety of tumors has been supported by extensive clinical evidence and results of studies on experimental mouse models. Therefore, when the malignant potential of tumors is considered, it will be necessary to evaluate the biologic properties of stromal cells, including CAFs and cancer cells.

Lung cancer is associated with high mortality and is the leading cause of cancer death in the world. Adenocarcinoma and squamous cell carcinoma (SqCC) are the two major histologic subtypes of non-small

cell lung cancer (NSCLC); however, the pathogenesis and biologic characteristics of the two subtypes differ. Although several reports have described the prognostic markers for adenocarcinoma, few reports have discussed the prognostic markers in patients with SqCC. We have reported that SqCC with a fibrous stroma exhibited a more invasive phenotype and was associated with a significantly poorer prognosis.[7] This finding suggests that the microenvironment created by both SqCC cells and the peritumoral fibroblasts may promote cancer aggressiveness.

Although surgical resection is considered the most effective therapy for patients with stage I disease, approximately 40% of patients with stage I disease die within 5 years. Furthermore, the survival benefit of adjuvant chemotherapy for patients with stage I disease remains controversial. Therefore, it is important to identify risk factors in patients with stage I disease to assess whether they would benefit from adjuvant chemotherapy.

Several molecular markers including CD44, carbonic anhydrase IX (CA-IX), laminin-5, CD10, and E-cadherin on cancer cells have been reported to have prognostic value in patients with NSCLC.[8-35] On the other hand, in regard to the CAFs, previous reports have identified a few prognostic markers in patients with NSCLC.[32,36-38] However, to date, there have been no reports on the prognostic significance of markers on CAFs in SqCC.

The purpose of this study was to identify the prognostic biologic markers of stage I SqCC by examination of the immunophenotypes of both cancer cells and CAFs. We examined the expressions of seven markers on cancer cells, including E-cadherin, laminin-5, podoplanin, c-MET, CA-IX, CD10, and CD44, which are reported to be of prognostic value in several types of cancer cells. To examine prognostic value of CAFs, podoplanin, CA-IX, and CD10 were selected, since these markers have been reported as prognostic markers in NSCLC.

Materials and Methods

Subjects

Between April 1994 and December 2006, a total of 194 patients with stage I lung SqCC underwent surgery with curative intent at the National Cancer Center Hospital East in Chiba, Japan. Among these patients, 52 were excluded because (1) they had past medical histories of malignant tumors within the previous 5-year period, and (2) satisfactory specimens were not available. Finally, 142 patients were included in this study. All specimens were collected after obtaining written informed consent from the patient, and the study was conducted with the approval of the Institutional Review Board of the National Cancer Center. IRB approval number of this study is 2010-095.

The tumors were staged according to the International Union Against Cancer TNM classification and were histologically subtyped and graded according to the third edition of the World Health Organization guidelines. The clinical data of the patients were obtained from the hospital charts. The mean age of the patients at the time of surgery was 66 years (range, 52-88 years), and the median follow-up time was 5.2 years. Overall survival was measured 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. The clinicopathologic characteristics of the patients and the results are summarized in Table 1.

Pathologic Studies

Surgical specimens were fixed with 10% formalin or methanol and embedded in paraffin. The tumors were cut at approximately 5-mm intervals, and serial 4-μm sections were stained with hematoxylin and eosin. The histologic diagnoses were based on the revised World Health Organization histologic classification. Tumor size was measured as the maximal diameter on the cut section of the lung. The pathologic stage was determined according to the classification of the Union Internationale Contre le Cancer.

Antibodies and Immunohistochemistry

Sections, 4-μm each, were cut from the paraffin blocks and mounted on salinized slides. Primary antibodies used in this study were summarized in e-Table 1. After antigen retrieval, the slides were immersed in a 0.3% hydrogen peroxide solution in methanol for 15 min to inhibit endogenous peroxidase activity. Individual slides were then incubated overnight at 4°C with different antibodies, and after extensive washing with phosphate-buffered saline, the smears were incubated with EnVision (Dako) for 1 h at room temperature. The color reaction was developed for 3 min 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.

We first selected three cutoff values (10%, 20%, and 50% of the cancer cells and CAFs) to define high and low staining. However, there were no differences in *P* value for 5-year survival rate except C-Met expression in cancer cells (data not shown). When cutoff value was chosen as 10% and 20%, the number of low-staining cases was too small in some antibodies (10%, four cases; 20%, eight cases; in CD44-low CAFs). Moreover, measurement of 50% of cancer cells and CAFs can be easily and reliably assessed. When ≥50% of the cancer cells or CAFs showed positive reaction, the case was classified as showing high expression.

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Table 1—*Univariate Prognostics Analysis of Clinicopathologic Factors*

Factors	No (%)	5-y Survival Rate, %	*P* Value
Age			
< 65 y	45 (32)	81.7	.024[a]
≥ 65 y	97 (68)	57.4	...
Median, 66 y			
Sex			
Male	125 (88)	65.2	...
Female	17 (12)	60.2	.65
Tumor diameter, mm			
≤ 30	69 (49)	72.5	...
> 30, ≤ 50	73 (51)	57.0	.036[a]
Pathologic stage			
IA	67 (47)	75.1	...
IB	75 (53)	55.2	.011[a]
Tumor location			
Central type	26 (18)	69.2	...
Peripheral type	116 (82)	69.8	.84
Histologic differentiation			
Well/moderately	97 (68)	62.3	...
Poor	45 (32)	69.7	.60
Pleural invasion			
Absence	111 (78)	66.0	...
Presence	31 (22)	59.3	.31
Vascular invasion			
Absence	69 (49)	70.0	.047[a]
Presence	73 (51)	59.3	...
Lymphatic permeation			
Absence	122 (86)	61.9	...
Presence	20 (14)	79.7	.11

N = 142. Log-rank test was used in comparison between the two groups.
[a]$P < 0.05$.

Statistical Analysis

The correlations between the antibody expressions in the cancer cells or CAFs and the overall survival times were evaluated by the log-rank test. The correlations between the antibody expressions in the cancer cells and CAFs were evaluated by Fisher exact test, as appropriate (e-Table 2). Survival curves were estimated by the Kaplan-Meier method, and differences in survival between subgroups were compared by the log-rank test. Multivariate analysis was conducted using the Cox proportional hazard model. *P* values < .05 were considered to be significant. A statistical analysis software (JMP, version 8) was used to perform the analyses.

Table 2—*Univariate Analysis of Biologic Markers, Cancer Cell*

Antibodies Expression	High, No. (%)	Low, No. (%)	5-y Survival Rate, %	*P* Value
E-cadherin	118 (83)	24 (17)	High: 70.1 Low: 38.8	< .001[a]
Laminin-5γ2	34 (24)	108 (76)	High: 51.5 Low: 69.0	.06
Podoplanin	63 (44)	79 (56)	High: 64.2 Low: 65.8	.76
C-MET	30 (21)	112 (79)	High: 76.0 Low: 61.8	.11
CA-IX	17 (12)	125 (88)	High: 53.8 Low: 66.1	.31
CD10	20 (14)	122 (86)	High: 60.1 Low: 65.4	.61
CD44	125 (88)	17 (12)	High: 71.2 Low: 70.6	.70

Log-rank test was used in comparison between the two groups. CA-IX = carbonic anhydrase IX.
[a]$P < .05$.

Results

Patient Characteristics

The median age of the 142 patients was 66 years (58-80 years). Among the 142 patients, 125 (88%) were men, and 17 (12%) were women. The number of patients with pathologic stage IA and IB disease were 67 (47%) and 75 (53%), respectively.

Univariate analysis of the clinicopathologic factors was performed to identify factors influencing the overall survival, and the log-rank test was used for comparison between the two groups. Patient age (> 65 years), tumor size, pathologic stage IB, and presence of vascular invasion were significantly correlated with a shorter survival time ($P = .024$, $P = .010$, $P = .019$, and $P = .047$, respectively) (Table 1).

Immunohistochemical Staining of the Cancer Cells and Prognostic Impact of the Immunophenotype

Univariate analyses were performed according to the Cox proportional hazard model to determine the

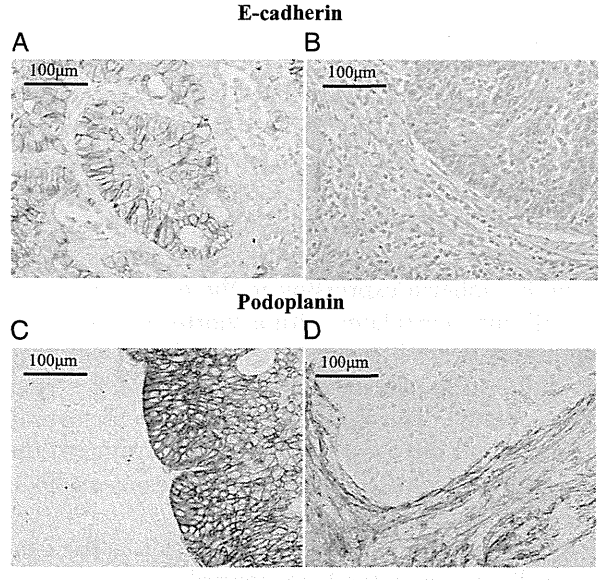

FIGURE 1. A, High E-cadherin expression in the cancer cells with a membranous staining pattern. B, Negative E-cadherin expression in the cancer cells. C, Negative podoplanin expression in the cancer-associated fibroblasts (CAFs) (the cancer cells exhibited high podoplanin expression). High podoplanin expression in cancer cells was observed in the periphery of the tumor nests. D, High podoplanin expression in the CAFs.

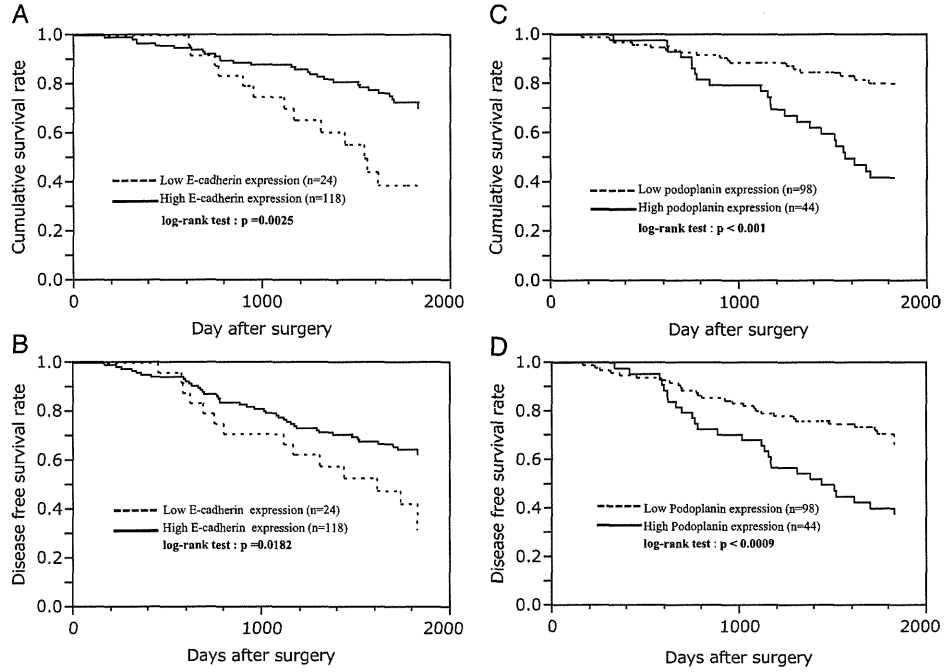

FIGURE 2. A, Kaplan-Meier curve for overall survival patients with pathologic stage I lung SqCC according to the expression status of E-cadherin in the cancer cells. High expression, solid line; low expression, dotted line. B, Kaplan-Meier curve for disease-free survival in patients with pathologic stage I lung SqCC according to the expression status of E-cadherin in the cancer cells. High expression, solid line; low expression, dotted line. C, Kaplan-Meier curve for overall survival in patients with pathologic stage I lung SqCC according to the expression status of podoplanin in the CAFs. High expression, solid line; low expression, dotted line. D, Kaplan-Meier curve for disease-free survival in patients with pathologic stage I lung SqCC according to the expression status of podoplanin in the CAFs. High expression, solid line; low expression, dotted line. SqCC = squamous cell carcinoma. See Figure 1 legend for expansion of other abbreviation.

prognostic value of the expression of each molecule in cancer cells (Table 2). Low E-cadherin expression was observed in 118 cases (83%) and high E-cadherin expression in 24 (17%). Figures 1A and 1B show representative results of E-cadherin expression in cancer cells. The 5-year survival rate of cases showing high E-cadherin expression was 70.0%, and that of the cases showing low E-cadherin expression was 38.5%. Low E-cadherin expression in the cancer cells was significantly correlated with a shorter survival time and short interval to recurrence compared with high E-cadherin in cancer cells ($P < .001$ and $P = .018$, respectively). The Kaplan-Meier curve for overall survival and disease-free survival according to the E-cadherin expression status in the cancer cells is shown in Figures 2A and 2B.

Cases with high laminin-5 expression in the cancer cells showed a tendency toward shorter survival times as compared with cases showing low laminin-5 expression; however, the difference was not significant ($P = .08$). The expression statuses of podoplanin, c-MET, CA-IX, CD10, and CD44 in the cancer cells had no prognostic impact.

Immunohistochemical Staining of CAFs and the Prognostic Impact of the Immunophenotype

High podoplanin expression in the CAFs was observed in 44 cases (31%). Figures 1C and 1D show representative cases to show the impact of podoplanin expression in the CAFs. The 5-year survival rate of the cases showing low podoplanin expression was 76.6%, whereas that of the patients showing high podoplanin expression in the cancer cells was 41.0%. High podoplanin expression in the CAFs has been demonstrated to show a significantly positive correlation with a shorter survival time and short interval to recurrence ($P < .001$ and $P < .0009$, respectively). The Kaplan-Meier curve for the overall and disease-free survivals according to the expression status of podoplanin expression in the CAFs are shown in Figures 2C and 2D. None of the other molecules examined in the CAFs showed any prognostic impact (Table 3).

Multivariate Analyses to Identify Factors Significantly Associated With the Prognosis

A multivariate analysis using the Cox proportional hazard model was performed to determine the

Table 3—*Univariate Analysis of Biologic Markers, Cancer-Associated Fibroblast*

Antibodies Expression	High, No. (%)	Low, No. (%)	5-y Survival Rate, %	*P* Value
Podoplanin	44 (31)	98 (69)	High: 42.0 Low: 76.6	<.001[a]
CA-IX	17 (12)	125 (88)	High: 70.1 Low: 65.0	.35
CD10	16 (11)	126 (89)	High: 62.5 Low: 64.9	.13
CD44	126 (89)	16 (11)	High: 71.2 Low: 70.6	.70

Log-rank test was used in comparison between the two groups. See Table 2 legend for expansion of abbreviation.
[a]$P < .05$.

prognostic usefulness of conventional pathologic factors and low E-cadherin expression in the cancer cells/high podoplanin expression in the CAFs (Table 4). Patient age and low E-cadherin expression in the cancer cells/high podoplanin expression in the CAFs were identified as significantly independent prognostic factors for the overall survival ($P = .0102$, $P = .0133$, and $P = .0010$, respectively).

Subgroup Analysis Using a Combination of the Expression Status of E-Cadherin in Cancer Cells and That of Podoplanin in CAFs

We divided the 142 patients into three groups, as follows: Group A, high E-cadherin expression in the cancer cells/low podoplanin expression in the CAFs; group B, high E-cadherin expression in the cancer cells/high podoplanin expression in the CAFs or low E-cadherin expression in the cancer cells/low podoplanin expression in the CAFs; group C, low E-cadherin expression in the cancer cells/high podoplanin expression in the CAFs.

The overall survival curves of the three groups are shown in Figure 3A. Group C showed a significantly shorter survival time as compared with group A ($P < .001$) or group B ($P < .01$). Representative figures of E-cadherin expression in the cancer cells and podoplanin expression in the CAFs of a group C case are shown in Figure 3B.

Discussion

This is the first report, to our knowledge, of examination of the prognostic significance of biologic markers in lung SqCC focusing on the characteristics of both cancer cells and CAFs. We showed that low E-cadherin expression in the cancer cells and high podoplanin expression in the CAFs were independent prognostic factors in stage I lung SqCC. Furthermore, cases fulfilling both criteria exhibited a significantly poorer prognosis. The current study also suggested the possibility that the microenvironment created by cancer cells with low E-cadherin expression and the surrounding CAFs with high podoplanin expression would be conducive to cancer cell survival, proliferation, and migration.

E-cadherin is a transmembrane glycoprotein, which is essential for calcium-dependent intercellular adhesion. It is widely accepted that decreased expression of E-cadherin as a cell adhesion molecule promotes detachment of cancer cells and increases the metastatic potential.[11,22] Our current study showed that low E-cadherin expression in cancer cells was significantly correlated with moderate/poor differentiation ($P = .0007$, data not shown) and worse prognosis than high E-cadherin expression in the cells, which is consistent with previous reports.[11,13,18,22]

Kawase et al[37] showed that high podoplanin expression in CAFs was correlated with a poor prognosis in cases of lung adenocarcinoma. Our results indicated a prognostic significance of high podoplanin expression in the CAFs also in lung SqCC. Recently, Hoshino et al[38] reported that podoplanin expressed on fibroblasts contributed functionally to proliferation of the lung adenocarcinoma cell line A549 in animal models. These results suggest the possibility that podoplanin on the CAFs plays some functionally important roles in the process of tumor progression in both adenocarcinoma and SqCC of the lung via cancer cell-CAF interactions. On the other hand, Yamanashi et al[39] reported that high podoplanin expression in colorectal CAFs was correlated with a better prognosis. This difference might be explained by differences in the functions of podoplanin-expressing CAFs in different organs.

Table 4—*Multivariate Data Analysis of Prognosis Factors (N = 142)*

Variable	Favorable	Unfavorable	Hazard Ratio	95% CI	Multivariate Data Analysis, *P* Value
Age	<65 y	≥65 y	2.63	1.238-6.478	.0102[a]
Vascular invasion	Absence	Presence	1.65	0.859-3.284	.1326
Tumor diameter	≤30 mm	>30, ≤50 mm	1.42	0.736-2.832	.2984
E-cadherin expression in cancer cells	High	Low	2.46	1.219-4.733	.0133[a]
Podoplanin expression in CAFs	Low	High	2.79	1.52-5.186	.0010[a]

Multivariate analysis was conducted using the Cox proportional hazard model. CAFs = cancer-associated fibroblasts. See Table 2 legend for expansion of other abbreviation.
[a]$P < .05$.

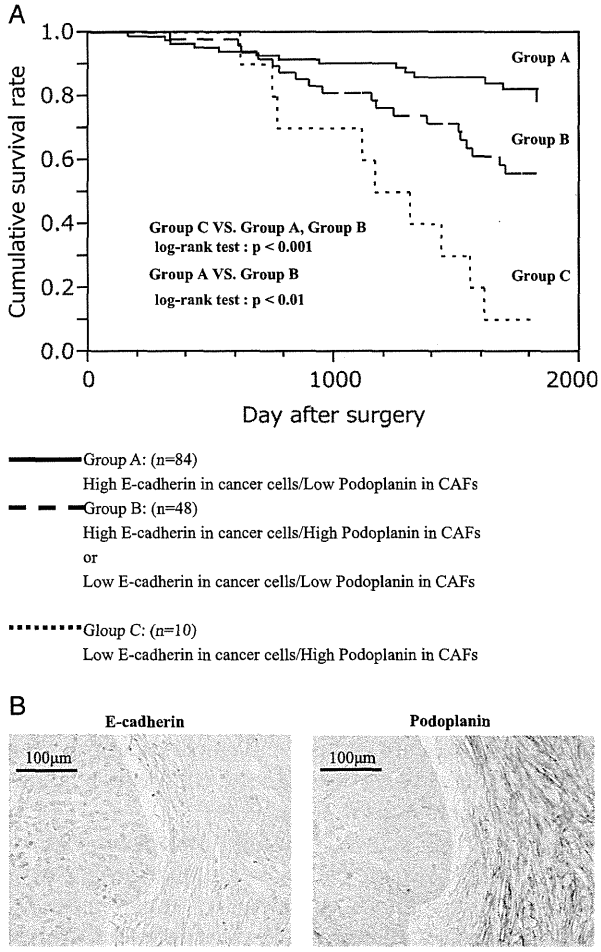

FIGURE 3. A, Kaplan-Meier curve for overall survival in patients with pathologic stage I lung SqCC according to the expression status of E-cadherin in the cancer cells and that of podoplanin in the CAFs. Group A: high E-cadherin expression in the cancer cells/low podoplanin expression in the CAFs; group B, high E-cadherin expression in the cancer cells/high podoplanin expression in the CAFs or low E-cadherin expression in the cancer cells/low podoplanin expression in the CAFs; group C, low E-cadherin expression in the cancer cells/high podoplanin expression in the CAFs. B, Representative case of group C. Left: no E-cadherin expression was found in the cancer cells; right: podoplanin expression was found in the CAFs. In this case, the cancer cells at the periphery of the tumor cell nests were weakly positive for podoplanin. See Figure 1 and 2 legends for expansion of abbreviations.

The results of multivariate analysis showed that age, low E-cadherin expression in cancer cells, and high podoplanin expression in CAFs were independent prognostic factors. Nakao et al[36] reported that the high expression of CA-IX in cancer cells and CAFs was an independent prognostic predictor in patients with lung adenocarcinoma. Furthermore, they showed that CA-IX expression on CAFs was a better predictor of the outcome than CA-IX expression on cancer cells. The current results also suggest that the prognostic value of molecular markers in CAFs could be as significant as that of the markers on cancer cells. Taking these observations into consideration, the malignant potential of cancer may have to be evaluated by the characteristics of not only the cancer cells but also the CAFs.

Laminin-5 is known to mediate the attachment, migration, and organization of cells into tissues during embryonic development and is regarded as a marker of the invasive activity of cancer cells.[20] Moriya et al[23] reported that patients with adenocarcinoma with high laminin-5 expression showed a poor prognosis. Our results also showed a tendency toward patients with a high laminin-5 expression in cancer cells showing a poor prognosis. Interestingly, high laminin-5 expression in the cancer cells was significantly correlated with high podoplanin expression in the CAFs ($P < .001$) (e-Table 2). Two possibilities may explain this phenomenon. First, high laminin-5 expression in the cancer cells can induce podoplanin expression in the CAFs via cell-cell interaction, and second, podoplanin expressed on the CAFs can increase the expression of laminin-5 on the cancer cells. Analysis of the molecular interaction between the cancer cells expressing laminin-5 and CAFs expressing podoplanin may provide a novel insight into cancer cell invasiveness.

In conclusion, the current study indicates that the immunophenotypes of CAFs could have prognostic significance independent of that of the cancer cells in patients with pathologic stage I lung SqCC. Our results may point to the critical need for more careful follow-up and individual additional treatments, including postoperative chemotherapy, in cases showing low E-cadherin expression in the cancer cells and high podoplanin expression in the CAFs, even those with early-stage lung carcinoma. Furthermore, considering the recent report that podoplanin in CAFs is the functional protein that is responsible for tumor progression, addition of anti-CAF therapy for patients with podoplanin-positive CAFs may enable a better response to the treatment of lung cancer.

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Dr Ono: contributed to the design and coordination of the study, preparing the manuscript, and read and approved the final manuscript.

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

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

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Dr Ikeda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Ochiai: contributed to revise the article for important intellectual content and read and approved the final manuscript.

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References

1. Ito TK, Ishii G, Chiba H, Ochiai A. The VEGF angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells. *Oncogene*. 2007;26(51):7194-7203.
2. Ishii G, Sangai T, Oda T, et al. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochem Biophys Res Commun*. 2003;309(1):232-240.
3. Ishii G, Ito TK, Aoyagi K, et al. Presence of human circulating progenitor cells for cancer stromal fibroblasts in the blood of lung cancer patients. *Stem Cells*. 2007;25(6):1469-1477.
4. Xing F, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front Biosci*. 2010;15:166-179.
5. Okusa Y, Ichikura T, Mochizuki H. Prognostic impact of stromal cell-derived urokinase-type plasminogen activator in gastric carcinoma. *Cancer*. 1999;85(5):1033-1038.
6. Korc M. Pancreatic cancer-associated stroma production. *Am J Surg*. 2007;194(4 suppl):S84-S86.
7. Takahashi Y, Ishii G, Taira T, et al. Fibrous stroma is associated with poorer prognosis in lung squamous cell carcinoma patients. *J Thorac Oncol*. 2011;6(9):1460-1467.
8. Zhao J, Sun Y, Zhang L. Preliminary study on the correlation between expression of CD44 in human non-small cell lung cancer and lymph node metastasis [in Chinese]. *Zhongguo Fei Ai Za Zhi*. 2000;3(2):115-117.
9. Zhao H, Fang J, Du G. Expression of standard and variant CD44 in human lung cancer [in Chinese]. *Zhonghua Jie He He Hu Xi Za Zhi*. 1998;21(9):541-543.
10. You J, Zhang X, Zhang J, Chen H, Liu Y, Sun L. Expression of thrombospondin-1 and CD44 in human lung cancer tissues and their prognostic significance [in Chinese]. *Zhongguo Fei Ai Za Zhi*. 2000;3(3):175-179.
11. Ucvet A, Kul C, Gursoy S, et al. Prognostic value of epithelial growth factor receptor, vascular endothelial growth factor, E-cadherin, and p120 catenin in resected non-small cell lung carcinoma [in Spanish]. *Arch Bronconeumol*. 2011;47(8):397-402.
12. Tokuhara T, Adachi M, Hashida H, et al. Neutral endopeptidase/CD10 and aminopeptidase N/CD13 gene expression as a prognostic factor in non-small cell lung cancer. *Jpn J Thorac Cardiovasc Surg*. 2001;49(8):489-496.
13. Tamura S, Shiozaki H, Miyata M, et al. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. *Br J Surg*. 1996;83(11):1608-1614.
14. Swinson DE, Jones JL, Richardson D, et al. Carbonic anhydrase IX expression, a novel surrogate marker of tumor hypoxia, is associated with a poor prognosis in non-small-cell lung cancer. *J Clin Oncol*. 2003;21(3):473-482.
15. Suzuki H, Onimaru M, Yonemitsu Y, Maehara Y, Nakamura S, Sueishi K. Podoplanin in cancer cells is experimentally able to attenuate prolymphangiogenic and lymphogenous metastatic potentials of lung squamoid cancer cells. *Mol Cancer*. 2010;9:287.
16. Suzuki H, Onimaru M, Koga T, et al. High podoplanin expression in cancer cells predicts lower incidence of nodal metastasis in patients with lung squamous cell carcinoma. *Pathol Res Pract*. 2011;207(2):111-115.
17. Salgia R. Role of c-Met in cancer: emphasis on lung cancer. *Semin Oncol*. 2009;36(2)(Suppl 1):S52-S58.
18. Qiu X, Yang X, Li Q, Wang E. A study of E-cadherin and beta-catenin expression and their correlation with prognosis of nonsmall cell lung carcinoma [in Chinese]. *Zhonghua Bing Li Xue Za Zhi*. 2002;31(4):318-321.
19. Ochiai S, Nakanishi Y, Mizuno K, et al. Expression of CD44 standard and CD44 variant 6 in human lung cancer [in Japanese]. *Nihon Kyobu Shikkan Gakkai Zasshi*. 1997;35(11):1179-1185.
20. Nishino T, Ishida T, Oka T, Yasumoto K, Sugimachi K. Prognostic significance of laminin in adenocarcinoma of the lung. *J Surg Oncol*. 1990;43(4):214-218.
21. Niki T, Kohno T, Iba S, et al. Frequent co-localization of Cox-2 and laminin-5 gamma2 chain at the invasive front of early-stage lung adenocarcinomas. *Am J Pathol*. 2002;160(3):1129-1141.
22. Myong NH. Reduced expression of E-cadherin in human non-small cell lung carcinoma. *Cancer Res Treat*. 2004;36(1):56-61.
23. Moriya Y, Niki T, Yamada T, Matsuno Y, Kondo H, Hirohashi S. Increased expression of laminin-5 and its prognostic significance in lung adenocarcinomas of small size. An immunohistochemical analysis of 102 cases. *Cancer*. 2001;91(6):1129-1141.
24. Lu ZQ, Li HG, Zhang HZ, Fan MJ, Shen XM, He XX. Expression and significance of CD44(+)ESA(+)CD24(-/low), stem cell markers for breast cancer, in non-small-cell lung carcinoma [in Chinese]. *Ai Zheng*. 2008;27(6):575-579.
25. Leung EL, Fiscus RR, Tung JW, et al. Non-small cell lung cancer cells expressing CD44 are enriched for stem cell-like properties. *PLoS ONE*. 2010;5(11):e14062.
26. Kristiansen G, Schlüns K, Yongwei Y, Dietel M, Petersen I. CD10 expression in non-small cell lung cancer. *Anal Cell Pathol*. 2002;24(1):41-46.
27. Kon-no H, Ishii G, Nagai K, et al. Carbonic anhydrase IX expression is associated with tumor progression and a poor prognosis of lung adenocarcinoma. *Lung Cancer*. 2006;54(3):409-418.
28. Kim SJ, Rabbani ZN, Vollmer RT, et al. Carbonic anhydrase IX in early-stage non-small cell lung cancer. *Clin Cancer Res*. 2004;10(23):7925-7933.
29. Ito T, Ishii G, Nagai K, et al. Low podoplanin expression of tumor cells predicts poor prognosis in pathological stage IB squamous cell carcinoma of the lung, tissue microarray analysis of 136 patients using 24 antibodies. *Lung Cancer*. 2009;63(3):418-424.
30. Ilie M, Mazure NM, Hofman V, et al. High levels of carbonic anhydrase IX in tumour tissue and plasma are biomarkers of poor prognostic in patients with non-small cell lung cancer. *Br J Cancer*. 2010;102(11):1627-1635.
31. Ichimura E, Maeshima A, Nakajima T, Nakamura T. Expression of c-met/HGF receptor in human non-small cell lung carcinomas in vitro and in vivo and its prognostic significance. *Jpn J Cancer Res*. 1996;87(10):1063-1069.

32. Gurel D, Kargi A, Karaman I, et al. CD10 expression in epithelial and stromal cells of non-small cell lung carcinoma (NSCLC): a clinic and pathologic correlation. *Pathol Oncol Res*. 2012;18(2):153-160.
33. Gumustekin M, Kargi A, Bulut G, et al. HGF/c-Met overexpressions, but not met mutation, correlates with progression of non-small cell lung cancer. *Pathol Oncol Res*. 2012;18(2):209-218.
34. Giatromanolaki A, Koukourakis MI, Sivridis E, et al. Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. *Cancer Res*. 2001;61(21):7992-7998.
35. Bremnes RM, Veve R, Gabrielson E, et al. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J Clin Oncol*. 2002;20(10):2417-2428.
36. Nakao M, Ishii G, Nagai K, et al. Prognostic significance of carbonic anhydrase IX expression by cancer-associated fibroblasts in lung adenocarcinoma. *Cancer*. 2009;115(12):2732-2743.
37. Kawase A, Ishii G, Nagai K, et al. Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. *Int J Cancer*. 2008;123(5):1053-1059.
38. Hoshino A, Ishii G, Ito T, et al. Podoplanin-positive fibroblasts enhance lung adenocarcinoma tumor formation: podoplanin in fibroblast functions for tumor progression. *Cancer Res*. 2011;71(14):4769-4779.
39. Yamanashi T, Nakanishi Y, Fujii G, et al. Podoplanin expression identified in stromal fibroblasts as a favorable prognostic marker in patients with colorectal carcinoma. *Oncology*. 2009;77(1):53-62.

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ORIGINAL ARTICLE

Prognostic impact of intratumoural microvascular invasion and microlymphatic permeation on node-negative non-small-cell lung cancer: which indicator is the stronger prognostic factor?†

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Abstract

OBJECTIVES: Microvascular invasion and microlymphatic permeation are indicators of microscopic tumour invasion into small vessels and have been considered to be powerful prognostic indicators for non-small-cell lung cancer (NSCLC). Several studies have suggested that these should be included in the TNM classification, but, there have been conflicting results regarding the prognostic impact of microvascular invasion and microlymphatic permeation. The aim of the current study was to clarify the prognostic impact of microvascular invasion and microlymphatic permeation on resected node-negative NSCLC by comparative analyses.

METHODS: We reviewed the data of 1039 consecutive patients with pathological size-based stage T1a-3N0M0 NSCLC who underwent lobectomy or greater resection between 1993 and 2005. The median follow-up period was 108 months. Microvascular invasion and microlymphatic permeation were identified by the Victoria blue–van Gieson staining. The overall survival was then analysed.

RESULTS: Microvascular invasion and microlymphatic permeation were observed in 358 (34.5%) and 205 (19.7%) of patients, respectively. Both microvascular invasion and microlymphatic permeation were more prevalent in non-adenocarcinoma and larger-sized tumours. The 5-year overall survival rate of the microvascular invasion-positive group and microlymphatic permeation-positive group were 69.2 and 84.6%, respectively, and the difference was statistically significant ($P = 0.002$). On multivariate analyses, microvascular invasion, but not microlymphatic permeation, was an independent prognostic factor (microvascular invasion, hazard ratio [HR] 1.648, $P = 0.001$; microlymphatic permeation, HR 1.138, $P = 0.588$). The 5-year overall survival rate of either the microvascular invasion- or microlymphatic permeation-positive T1a-b group was significantly lower than that of the corresponding double-negative (dn) T1a-b group (dnT1a-b, 93.7%; microvascular invasion-positive T1a-b, 85.2%, $P < 0.001$; microlymphatic permeation-positive T1a-b, 85.4%, $P = 0.014$), and overlapped to that of the dnT2a group (84.8%). However, in the T2a-b group, only microvascular invasion-positive T2a-b patients showed significantly lower overall survival than dnT2a-b patients, and their overall survival overlapped that of dnT3 patients (dn T2a-b, 83.5%; microvascular invasion-positive T2a-b, 60.6%, $P < 0.001$; dnT3, 53.8%; $P = 0.316$). The 5-year overall survival of microlymphatic permeation-positive T2a-b patients (86.2%) did not statistically differ from that of dnT2a-b patients ($P = 0.856$).

CONCLUSIONS: Microvascular invasion and microlymphatic permeation have different impact on survival, and microvascular invasion rather than microlymphatic permeation is a strong prognostic factor in resected node-negative NSCLC. Microvascular invasion and microlymphatic permeation should be examined separately by elastic staining.

Keywords: Non-small-cell lung cancer • Microvascular invasion • Microlymphatic permeation • Prognostic factor • Staging system

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide. The best treatment for early-stage non-small-cell lung cancer (NSCLC) is surgical resection. Within stage I disease, however, some patient characteristics are associated with worse survival, and many researchers have tried to identify the relevant prognostic factors. Microvascular invasion (MVI) and microlymphatic permeation (MLP) have been reported to be powerful prognostic indicators for resected NSCLC [1–8], and some investigators have suggested that these factors should be reflected in the TNM staging system. The most recent (7th) edition of the TNM classification of lung cancer dose not include MVI or MLP in the staging system, but has described both factors as 'optional descriptors' which deserved further investigation [9]. However, there have been conflicting reports regarding both. Some

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investigators have reported that only MLP, but not MVI, was significantly related to worse survival [6, 10, 11], whereas others stated opposite results [2, 5]. Some authors considered both blood vessel invasion (true MVI) and lymphatic permeation (MLP) to be MVI [4, 12], whereas others differentiate between blood vessels and lymphatic channels. Therefore, it remains unknown which, of MVI and MLP, has more impact on survival, and whether these factors should be separately examined. In this study, we attempted to clarify the clinicopathological features and prognostic impact on survival of MVI- and MLP-positive tumours in completely resected node-negative NSCLC by comparative analyses.

PATIENTS AND METHODS

Patient selection

A total of 2132 consecutive patients with NSCLC underwent pulmonary resection at our institution between 1993 and 2005. Among them, a total of 1039 patients with pathological T1a-3N0M0 who underwent complete resection of greater than lobectomy were included in this retrospective study. This study was approved by the institutional review board of our institution in 2010, and the need to obtain written informed consent was waived. Patients with the following characteristics were excluded: incomplete resection; neoadjuvant therapy; limited resections; T4, N1-3 and M1 disease and non-T-size-based T2a/T3 disease such as visceral pleural invasion (VPI) and chest wall invasion.

Histopathological examination

The surgical specimens were fixed in 10% formalin and underwent routine histopathological workup with paraffin embedding. Sections (4-μm thick) were cut and stained with haematoxylin and eosin (H&E). Victoria blue–van Gieson (VVG) staining to visualize elastic fibres was also routinely performed in all sections containing tumour cells to evaluate MVI and pleural invasion. MVI was determined to be positive if conspicuous clusters of tumour cells were present inside the microscopic lumen with a VVG-stained elastic layer in any section (Fig. 1A and B). MLP was considered to be positive when tumour cells were floating in VVG-negative, thin endothelial-lined channels with no supporting smooth muscles or elastic fibres in any section (Fig. 1C and D). As the artifactual spaces around the cancer nests were often indistinguishable from lymphatic vessels containing tumour emboli, when floating tumour cells were identified in lumens within the bronchovascular bundle, subpleural or interlobular pleural spaces, lymphatic permeation was concluded to be present. When we were not confident that the findings represented lymphatic permeation, we did not record the case as positive. Pathological evaluation using H&E and VVG staining was performed by two or more pathologists who were blinded to the clinical outcome, and the results were retrospectively collected.

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 computed tomography (CT) scans of the chest and abdomen, brain magnetic resonance imaging and bone scintigraphy were performed. Since

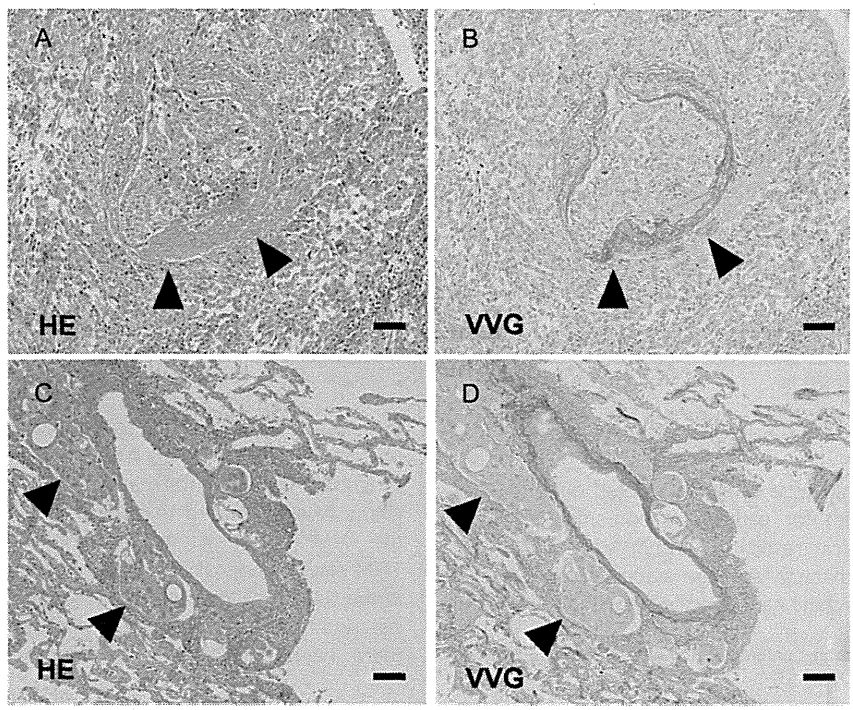

Figure 1: Representative pathological findings of MVI and MLP. MVI within a tumour nest with (**A**) H&E staining and (**B**) VVG staining. MLP within a tumour nest with (**C**) H&E staining and (**D**) VVG staining. Scale bar indicates 100 μm.

2004, integrated positron emission tomography and CT (PET-CT) was also performed for selected patients.

Statistical analysis

Two-category comparison was performed by the Pearson's χ^2 test. Overall survival (OS) was measured from the date of surgery to the date of death from any cause or last follow-up. The median follow-up period was 108 months (range 1-208). All cumulative survival rates were estimated using the Kaplan-Meier method, and differences in variables were evaluated using the log-rank test. Cox proportional hazards multivariate models were used to identify the independent prognostic factors. A *P*-value ≤0.05 was considered to represent statistically significant differences. The analyses were performed using statistical software JMP 8.0 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA).

Table 1: Patient characteristics of the study population

	N	Percent
Age, median (range) (year)	66 (20-89)	
Men/women	601/438	57.8/42.2
Preoperative CEA level (ng/ml)		
Median (range)	3.5 (0.7-739.2)	
Histology		
Adenocarcinoma	747	71.9
Squamous cell carcinoma	221	21.3
Large cell carcinoma	47	4.5
Others	24	2.3
p-T factors		
T1a (≤2 cm)	354	34.1
T1b (2-3 cm)	277	26.7
T2a (3-5 cm)	315	30.3
T2b (5-7 cm)	62	5.9
T3 (>7 cm)	31	3.0

CEA: carcinoembryonic antigen.

RESULTS

The patient characteristics are shown in Table 1. The median age was 66 years (range 20-89). There were 601 (57.8%) men and 438 (42.2%) women. Adenocarcinoma was the most common histology (71.9%). The distribution of p-T factors (tumour size) was as follows: T1a (≤2 cm), 354 (34.1%); T1b (2-3 cm), 277 (26.7%); T2a (3-5 cm), 315 (30.3%); T2b (5-7 cm), 62 (5.9%); T3 (>7 cm), 31 (3.0%). Overall, MVI and MLP were observed in 358 (34.5%) and 205 (19.7%) patients, respectively. MVI alone (MVI +/MLP-), MLP alone (MVI-/MLP+) and both MVI and MLP (MVI +/MLP+) were observed in 231 (22.2%), 78 (7.5%) and 127 (12.2%) patients, respectively. The prevalence of MVI was significantly higher in patients >70 years ($P < 0.001$), male patients ($P < 0.001$), patients with non-adenocarcinomas ($P < 0.001$) and patients with large-sized tumours ($P < 0.001$). The prevalence of MLP showed a similar trend. MLP was significantly prevalent in male patients ($P = 0.05$), patients with non-adenocarcinoma ($P = 0.006$) and large-sized tumours ($P < 0.001$) (Table 2). During the follow-up, 191 (18.4%) had recurrence (Table 3). The initial site of recurrence was locoregional in 89 (46.6%) patients and distant in 102 (53.4%) patients. The patients with MVI-positive (MVI +/MLP- and MVI+/MLP+) tumours developed more recurrences (31.2 and 27.6%) than the patients with only MLP-positive (MVI -/MLP+) tumours (18.0%). Among the patients with recurrence, distant metastases were noted more frequently in patients with MVI-positive (MVI+/MLP- and MVI+/MLP+) tumours (58.3 and 68.6%) compared with patients with only MLP-positive (MVI -/MLP+) tumours (28.6%). The OS curves according to MVI and MLP status are shown in Figure 2. The patients with MLP only tumours (MVI-/MLP+) had significantly lower OS rates than those with neither factor (MVI-/MLP-) ($P < 0.001$). The patients with MVI only tumours (MVI+/MLP-) had much lower OS rates compared with those with MLP only tumours (MVI-/MLP+) ($P = 0.002$). The 5-year OS rate was 90.7% in patients with neither factor (MVI-/MLP-), 84.6% in patients with only MLP (MVI-/MLP+), 69.2% in patients with only MVI (MVI+/MLP-) and 58.3% in patients with both MVI and MLP (MVI+/MLP+). Univariate analysis by using MVI+, MLP+, MVI+/MLP+ and other

Table 2: Correlation between clinicopathological factors and the prevalence of MVI and MLP

Factors	MVI+, *n* (%)	*P*-value	MLP+, *n* (%)	*P*-value
Age (year)		<0.001		0.416
≤70 (*n* = 667)	202 (30.2)		137 (20.5)	
>70 (*n* = 372)	156 (41.9)		68 (18.3)	
Gender		<0.001		0.050
Male (*n* = 601)	257 (42.8)		131 (21.8)	
Female (*n* = 438)	101 (23.1)		74 (16.9)	
Preoperative CEA level (ng/ml)[a]		<0.001		0.281
≤5 (*n* = 688)	199 (28.9)		130 (18.9)	
>5 (*n* = 342)	158 (46.2)		75 (21.9)	
Histology		<0.001		0.006
Adenocarcinoma (*n* = 747)	187 (25.0)		131 (17.5)	
Non-adenocarcinoma (*n* = 292)	171 (58.6)		74 (25.3)	
p-T factors		<0.001		<0.001
T1a-T1b (*n* = 631)	121 (19.2)		118 (18.7)	
T2a-T3 (*n* = 408)	237 (58.1)		117 (28.7)	

MVI: microvascular invasion; MLP: microlymphatic permeation; CEA: carcinoembryonic antigen.
[a]Nine patients are excluded due to an unknown preoperative CEA level.

Table 3: Initial site of recurrence by MVI and MLP status

	No. of patients	No. of recurrences (%)	Initial site of recurrence	
			Locoregional (%)	Distant (%)
Overall	1039	191 (18.4)	89 (46.6)	102 (53.4)
MVI+/MLP−	231	72 (31.2)	30 (41.7)	42 (58.3)
MVI−/MLP+	78	14 (17.9)	10 (71.4)	4 (28.6)
MVI+/MLP+	127	35 (27.6)	11 (31.4)	24 (68.6)

MVI: microvascular invasion; MLP: microlymphatic permeation.

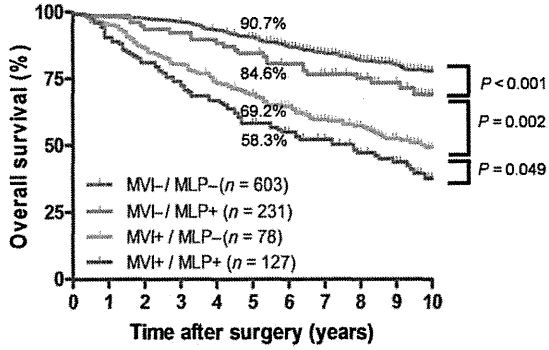

Figure 2: Overall survival curves of patients stratified according to MVI or MLP status.

pathological factors revealed that tumour size, histology and pleural invasion, in addition to MVI+, MLP+ and MVI+/MLP+, were significant prognostic factors. The 5-year OS rate was significantly lower in patients with MVI-positive (65.3%) compared with MVI-negative tumours (89.8%, $P < 0.001$), patients with MLP-positive (68.3%) relative to MLP-negative tumour (84.6%, $P < 0.001$), patients with MVI+/MLP+ (58.3%) compared with other population (MVI−/MLP−, MVI+/MLP− and MVI−/MLP+) (84.6%, $P < 0.001$), patients with large-sized tumour (>3 cm, 67.4%) compared with small-sized tumour (≤3 cm, 90.5%, $P < 0.001$), patients with non-adenocarcinoma (65.2%) compared with adenocarcinoma (87.7%, $P < 0.001$) and patients with tumours having pleural invasion (55.6%) compared with tumours without pleural invasion (85.5%, $P < 0.001$). Multivariate analysis demonstrated that a tumour size of >3 cm (HR 1.976, $P < 0.001$), non-adenocarcinoma histology (HR 1.757, $P < 0.001$), pleural invasion (HR 1.438, $P = 0.001$) and MVI+ (HR 1.648, $P < 0.001$) were independent poor prognostic factors associated with survival (Table 4). Neither MLP+ nor MVI+/MLP+ were statistically significant poor prognostic factors (HR 1.138, $P = 0.588$; HR 1.149, $P = 0.618$). We analysed the OS of groups of T1a-b and T2a-b patients stratified by different T-size categories (Fig. 3A and B). To evaluate the individual prognostic impact of both MVI and MLP, the patients with tumours which were double positive for both MVI and MLP ($n = 127$) were excluded from this analysis. In the T1a-b category, the presence of either MVI or MLP had a statistically significant negative prognostic impact on survival. Both the MVI-positive and MVI-negative T1a-b groups showed significantly lower OS rates than the double-negative (dn) T1a-b group (5-year OS: dnT1a-b, 93.7%; MVI-positive T1a-b, 85.2%, $P < 0.001$; MLP-positive T1a-b, 85.4%, $P = 0.014$), and showed similar OS to the dnT2a group (84.8% vs MVI-positive T1a-b, $P = 0.615$; vs MLP-positive T1a-b, $P = 0.450$) (Fig. 3A). On the other hand, in the T2a-b category, only MVI-positive T2a-b showed significantly lower OS rates than the dnT2a-b group, similar to the dnT3 group (5-year OS: dn T2a-b, 83.5%;

Table 4: Multivariate analysis of overall survival

Variable	HR	95% CI	*P*-value
Tumour size			
≤3 cm	1		
>3 cm	1.976	1.503–2.596	<0.001
Histology			
Adenocarcinoma	1		
Non-adenocarcinoma	1.756	1.381–2.233	<0.001
MVI+			
Negative	1		
Positive	1.648	1.220–2.222	<0.001
MLP+			
Negative	1		
Positive	1.138	0.699–1.768	0.588
MVI+/MLP+			
Negative	1		
Positive	1.149	0.673–2.024	0.618
Pleural invasion			
Negative	1		
Positive	1.438	1.070–1.929	0.001

HR: hazard ratio; CI: confidence interval; MVI: microvascular invasion; MLP: microlymphatic permeation.

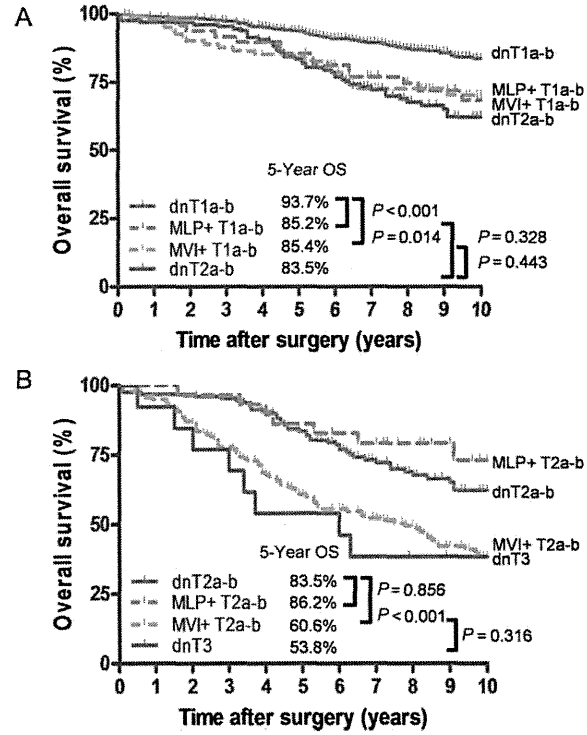

Figure 3: Overall survival curves of patients in the T1a-b (**A**) and T2a-b (**B**) groups stratified according to MVI or MLP status.

MVI-positive T2a-b, 60.6%, $P < 0.001$; dnT3, 53.8%; $P = 0.316$). The MLP-positive T2a-b group showed statistically similar survival (5-year OS, 86.2%) to the dnT2a-b group ($P = 0.856$) (Fig. 3B).

DISCUSSION

The importance of MVI and MLP as prognostic factors for resected NSCLC has been repeatedly reported by many investigators, including in our previous report [1–8, 10, 12]. Although, both MVI and MLP are indicators of microscopic tumour invasion into small vessels and are considered to be stages leading to tumour metastasis, it remains unknown which factor has more impact on survival because many studies have focussed only on either MVI or MLP. In this study, we performed a comparative survival analysis of patients with both MVI and MLP. The results indicated that the MVI-positive population had worse OS than the MLP-positive population, and multivariate analysis revealed that MVI, but not MLP, was an independent prognostic factor. When the presence or absence of MVI and MLP was incorporated in the TNM staging system, patients with MVI had significantly lower OS rate than the corresponding MVI-negative population in the T1a through T2b groups. The OS of patients in the T1a-b/MVI+ and T2a-b/MVI+ was statistically worse than that of patients in the dnT1a-b and dnT2a-b groups, and similar to that of patients in the dnT2a and dnT3 groups, respectively. On the other hand, the OS of the T1a-b/MLP+ group was worse than that of the dnT1a-b group and similar to that of the dnT2a group, but, the OS of the T2a-b/MLP+ group did not differ from that of the dnT2a-b group. Several studies have reported that MVI, but not MLP, is a poor prognostic factor of survival in patients with p-stage I NSCLC. Macchiarini *et al.* [13] reported that MVI was a predictor of postoperative recurrence and a poor prognostic factor in p-stage IA NSCLC patients. More recently, Shoji *et al.* [2] examined 217 p-stage IA NSCLC patients and demonstrated that MVI, but not MLP, was an independent prognostic factor. The results of the current study, which included a large number of patients with not only stage I but also higher stages, were similar to those of these previous studies, and indicate that the presence of MVI has a stronger prognostic impact on survival than MLP in patients with node-negative NSCLC. This further indicates that tumour cells from node-negative NSCLC may metastasize preferentially via the blood vessels rather than the lymphatic channels, and thus MVI can be considered stronger prognostic factor. Several investigators have considered MVI to be a combination of blood vessel invasion and MLP [3, 12]. However, as each has a different prognostic impact on survival, MVI and MLP should be examined separately. The method of differentiating between MVI and MLP is important. We usually use H&E and VVG stains on all tumours. Elastic stains including VVG staining provide the reliable detection and differentiation of MVI and MLP. MVI can be clearly differentiated from MLP by elastic stains because only the vascular walls contain elastic fibres. The reported detection rates of MVI and MLP in pathological stage I tumours without any elastic stains were 11–16 and 0–3%, respectively [14, 15]. In contrast, detection rates for MVI and MLP with elastic stains were 27–56 and 40–52%, respectively [10, 13, 16]. Generally, the reported detection rates of MVI and MLP have been higher in studies which used elastic stains to evaluate MVI. Elastic staining enables pathologists to identify MVI more precisely and to distinguish MVI from MLP. In the latest (7th) edition of the TNM classification, VPI is clearly defined, and elastic staining has been considered the optimal method of evaluating VPI [17]. Therefore, the routine use of elastic staining in the pathological examination of lung cancer is recommended, not only for VPI determination but also for identifying and differentiating MVI and MLP. However, in identifying MLP, the artificial spaces around the cancer nests are sometimes indistinguishable from MLP [18]. Recently, antibodies against D2-40 and podoplanin have been reported to be useful for identifying lymphatic vessels in various human cancers including NSCLC [19, 20]. Both antipodoplanin and D2-40 antibodies have a sensitivity and specificity for lymphatic endothelium of >95% [19]. Further studies are required to establish standardized criteria for the diagnosis of MLP.

The current data demonstrated that the MVI-positive population had significantly worse OS from the T1a group through the T2b group. Moreover, recent meta-analysis has shown survival benefit from cisplatin-based adjuvant chemotherapy in patients with stage II or higher NSCLC (LACE). A large adjuvant chemotherapy trial and meta-analysis conducted in Japan revealed that adjuvant chemotherapy with oral uracil-tegaful (UFT) had a survival benefit for stage IB adenocarcinoma patients, and demonstrated a tendency to improve survival even in patients with T1bN0M0. From the results of these studies, T1b-2aN0M0 and T2bN0M0 tumours may be candidates for adjuvant oral UFT and adjuvant cisplatin-based chemotherapy, respectively. In the current study, not only T1b/MVI+ but also T1a/MVI+ subgroups showed a significantly worse prognosis, consistent with the dnT2a subgroup. The T2a-b/MVI+ subgroup also showed significantly worse survival, similar to the dnT3 subgroup. Therefore, we suggest that T1a-b/MVI+ and T2a-b/MVI+ should be upstaged to T2a and T3, respectively. Patients with T1aN0M0/MVI+ and T2aN0M0/MVI+ may be good candidates for adjuvant oral UFT and adjuvant cisplatin-based chemotherapy, respectively. However, a limitation of the present study is that our proposal is incomplete, and must be verified by internationally collected multi-institutional data. Moreover, we recently reported the importance of MVI location (i.e. intratumoural or extratumoural) [21]. Among 1000 consecutive patients with NSCLC, intratumoural MVI (v1) and extratumoural MVI (v2) were identified in 428 (42.8%) and 32 (3.2%) patients, respectively. Although the v2 group was small, but showed significantly worse OS than the v1 group (5-year OS: v1, 55.9%; v2, 44.0%; $P = 0.010$). The location data of MVI should be also collected internationally for future validation.

In conclusion, the results of this study indicate that MVI and MLP have differing impacts on survival, and that MVI, rather than MLP, is a potent prognostic factor in resected node-negative NSCLC. MVI and MLP should be examined separately by elastic stains, and further data should be collected internationally, for consideration for the next revision of the TNM staging system.

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REFERENCES

[1] 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.

[2] Shoji F, Haro A, Yoshida T, Ito K, Morodomi Y, Yano T *et al.* Prognostic significance of intratumoral blood vessel invasion in pathologic stage IA non-small cell lung cancer. Ann Thorac Surg 2010;89:864–9.

[3] Tsuchiya T, Hashizume S, Akamine S, Muraoka M, Honda S, Tsuji K *et al.* Upstaging by vessel invasion improves the pathology staging system of non-small cell lung cancer. Chest 2007;132:170–7.

[4] Poncelet AJ, Cornet J, Coulon C, Collard P, Noirhomme P, Weynand B. Intra-tumoral vascular or perineural invasion as prognostic factors for long-term survival in early stage non-small cell lung carcinoma. Eur J Cardiothorac Surg 2008;33:799–804.

[5] Bodendorf MO, Haas V, Laberke HG, Blumenstock G, Wex P, Graeter T. Prognostic value and therapeutic consequences of vascular invasion in non-small cell lung carcinoma. Lung Cancer 2009;64:71–8.

[6] Harada M, Hato T, Horio H. Intratumoral lymphatic vessel involvement is an invasive indicator of completely resected pathologic stage I non-small cell lung cancer. J Thorac Oncol 2010;6:48–54.

[7] Naito Y, Goto K, Nagai K, Ishii G, Nishimura M, Yoshida J *et al.* Vascular invasion is a strong prognostic factor after complete resection of node-negative non-small cell lung cancer. Chest 2010;138:1411–7.

[8] Maeda R, Yoshida J, Ishii G, Hishida T, Nishimura M, Nagai K. Prognostic impact of intratumoral vascular invasion in non-small cell lung cancer patients. Thorax 2010;65:1092–8.

[9] International Association for the Study of Lung Cancer. Staging Handbook in Thoracic Oncology. Orange Park, FL: Editorial Rx Press, 2009.

[10] Brechot JM, Chevret S, Charpentier MC, Appere de Vecchi C, Capron F, Prudent J *et al.* Blood vessel and lymphatic vessel invasion in resected nonsmall cell lung carcinoma: correlation with TNM stage and disease free and overall survival. Cancer 1996;78:2111–8.

[11] Goldstein NS, Mani A, Chmielewski G, Welsh R, Pursel S. Prognostic factors in T1 N0 M0 adenocarcinomas and bronchioloalveolar carcinomas of the lung. Am J Clin Pathol 1999;112:391–402.

[12] Ruffini E, Asioli S, Filosso PL, Buffoni L, Bruna MC, Mossetti C *et al.* Significance of the presence of microscopic vascular invasion after complete resection of stage I-II pT1-T2N0 non-small cell lung cancer and its relation with T-size categories: did the 2009 7th edition of the TNM staging system miss something? J Thorac Oncol 2011;6:319–26.

[13] Macchiarini P, Fontanini G, Hardin MJ, Chuanchieh H, Bigini D, Vignati S *et al.* Blood vessel invasion by tumor cells predicts recurrence in completely resected T1 N0 M0 non-small-cell lung cancer. J Thorac Cardiovasc Surg 1993;106:80–9.

[14] Harpole DH Jr, Herndon JE 2nd, Young WG Jr, Wolfe WG, Sabiston DC Jr. Stage I nonsmall cell lung cancer. A multivariate analysis of treatment methods and patterns of recurrence. Cancer 1995;76:787–96.

[15] Poleri C, Morero JL, Nieva B, Vazquez MF, Rodriguez C, de Titto E *et al.* Risk of recurrence in patients with surgically resected stage I non-small cell lung carcinoma: histopathologic and immunohistochemical analysis. Chest 2003;123:1858–67.

[16] 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.

[17] Travis WD, Brambilla E, Rami-Porta R, Vallieres E, Tsuboi M, Rusch V *et al.* Visceral pleural invasion: pathologic criteria and use of elastic stains: proposal for the 7th edition of the TNM classification for lung cancer. J Thorac Oncol 2008;3:1384–90.

[18] Saijo T, Ishii G, Ochiai A, Hasebe T, Yoshida J, Nishimura M *et al.* Evaluation of extratumoral lymphatic permeation in non-small cell lung cancer as a means of predicting outcome. Lung Cancer 2007;55:61–6.

[19] Evangelou E, Kyzas PA, Trikalinos TA. Comparison of the diagnostic accuracy of lymphatic endothelium markers: Bayesian approach. Mod Pathol 2005;18:1490–7.

[20] Kadota K, Huang CL, Liu D, Ueno M, Kushida Y, Haba R *et al.* The clinical significance of lymphangiogenesis and angiogenesis in non-small cell lung cancer patients. Eur J Cancer 2008;44:1057–67.

[21] Shimada Y, Ishii G, Hishida T, Yoshida J, Nishimura M, Nagai K. Extratumoral vascular invasion is a significant prognostic indicator and a predicting factor of distant metastasis in non-small cell lung cancer. J Thorac Oncol 2010;5:970–5.

APPENDIX. CONFERENCE DISCUSSION

Dr F. Detterbeck *(New Haven, CT, USA)*: I have just a general comment. I think that we have to be careful with defining additional prognostic factors. It is always easy to take a set of patients and find something of statistical significance. It is often harder to repeat that in other studies. We certainly have many examples of prognostic factors that then are controversial in various studies and many fewer factors that end up being really valid. I think it is important work, but we have to look at it in a broader context.

One of my questions is, do you think that you have a better way of defining this that may be the key to resolving some of the controversies? My impression has been that the way that microvascular lymphatic invasion has been defined in other studies has been somewhat variable and not as consistent and that that is part of the reason why we haven't really been able to sort that out as well as we would like. Do you think this is potentially a better way, using elastic stains and as you have defined it?

Dr Hishida: This is a difficult question. There are many controversies about the different definitions of microvascular invasion and microlymphatic permeation. There are many controversies regarding the definition. So to resolve these issues, we need a consensus on what is the exact definition of the correct positive indicator. So for us, probably we need a consensus meeting to define exactly the definition.

Dr Detterbeck: Agree, and that would be somewhat of a comment of mine as well. There are many studies where people recommend that their finding should be part of the new staging system when it is revised again in 2017, but I think we have to be a little bit careful about that, because, remember, the stage classification, the accepted things were done because they were consistent in various studies and various regions and various histologic types and so forth, and I think we have to be more careful about recommending that. Maybe a good recommendation would be that this be considered in arriving at a consensus of how to define this. It might be a better first start.

Dr G. Veronesi *(Milan, Italy)*: Do you think that the different prognostic impact of the two variables adenocarcinoma and non-adenocarcinoma tumours may be related to a stage bias or were they independent factors not related to the stage? I mean, maybe adenocarcinoma were diagnosed at an earlier stage.

Dr Hishida: Probably, I think so, yes. But other studies revealed a different result. Adenocarcinoma had more chance of having microvascular invasion than microlymphatic permeation. But in my study, non-adenocarcinoma had a higher percentage of a positive rate of microvascular invasion than microlymphatic permeation. The reason is that adenocarcinoma treated in Japan, including many early stages, was comprised of BAC, bronchoalveolar carcinoma, and showing ground-glass opacity on CT scan. That's why, I think.