Table 5: Five-year overall survival rates of patients according to interval between gastric cancer and lung cancer

Interval (years)	*N*	5-year OS (%)	*P*-value[a]
≤1 vs >1	31/69	68.3 vs 79.8	0.897
≤2 vs >2	39/61	77.2 vs 75.9	0.965
≤3 vs >3	49/51	78.7 vs 74.7	0.519
≤5 vs >5	62/38	75.9 vs 77.2	0.9

OS: overall survival.
[a]Log-rank test.

Several reports [3, 13, 14] have suggested that the incidence of early-stage lung cancer is higher in patients with previous malignancy because of follow-up assessments after treatment for malignant tumours. Quadrelli *et al.* [3] described that patients with a previous malignancy were more frequently diagnosed at Stage I than those without a previous malignancy. One study found a 72% likelihood of developing Stage I + II lung cancer as a second primary tumour [13]. However, the present study found no significant differences in the clinical stages of lung cancer between the PGC and NGC groups. This is because about 40% patients with a history of gastric cancer had been surgically treated for lung cancer over 5 years after undergoing treatment for gastric cancer. These findings indicate that the frequency of routine follow-up was insufficient for many of these patients.

We found that the interval between gastric cancer and lung cancer did not influence the prognosis. A previous multivariate analysis [13] also found that the disease-free interval between the appearance of the first and the primary tumours does not significantly impact the survival of patients with lung cancer as a second primary malignancy. Thus, a previous controllable malignancy seems not to substantially influence the prognosis of lung cancer.

We also found a higher ratio of older and male patients in the PGC, than in the NGC group, which was consistent with published findings [1, 2, 11, 15]. Since cancer incidence increases with age, patients with a second primary malignancy are often older [1, 2, 11]. The incidence of secondary primary cancers is high in patients with lung, colorectal, hepatic and gastric cancers that are common among Japanese males [16]. Information about smoking was not available, but we are aware that smoking is an important factor in the development of lung and gastric cancers [17], and it is a potential risk factor for the development of a second tumour [18, 19].

The present study has some limitations. The retrospective design is one and another is the selection bias imposed by not including patients whose surgeons found no indication for surgically treating lung cancer due to the patient having had previous gastric cancer. Cox regression analysis did not reveal previous gastric cancer as a significant factor. Regardless, our findings suggested that if surgeons considered that a previous gastric cancer had been controllable, then it minimally influenced the prognosis of patients with lung cancer. However, the upper limit of the 95% CI was broad, and we should interpret this conclusion to allow the exclusion of only the effect of a history of gastric cancer history beyond a HR of 1.92.

In summary, we showed that a history of treatment for gastric cancer had low impact on postoperative survival after complete surgical resection of a second lung cancer and that no factor associated with gastric cancer influenced the surgical outcomes of lung cancer. Curative surgery for lung cancer should be recommended when gastric cancer has been curatively treated regardless of the interval between the onset of both types of cancer. Furthermore, patients with Stage I gastric cancer in particular might be considered suitable for inclusion in clinical trials. Patients with previous gastric cancer and other types of cancer should be prospectively investigated in the future.

Conflict of interest: none declared.

REFERENCES

[1] Pages PB, Mordant P, Cazes A, Grand B, Foucault C, Dujon A *et al.* Prognosis of lung cancer resection in patients with previous extra-respiratory solid malignancies. Eur J Cardiothorac Surg 2013;44:534–8.
[2] Aguilo R, Macia F, Porta M, Casamitjana M, Minguella J, Novoa AM. Multiple independent primary cancers do not adversely affect survival of the lung cancer patient. Eur J Cardiothorac Surg 2008;34:1075–80.
[3] Quadrelli S, Lyons G, Colt H, Chimondeguy D, Silva C. Lung cancer as a second primary malignancy: increasing prevalence and its influence on survival. Ann Surg Oncol 2009;16:1033–8.
[4] Massard G, Ducrocq X, Beaufigeau M, Elia S, Kessler R, Herve J *et al.* Lung cancer following previous extrapulmonary malignancy. Eur J Cardiothorac Surg 2000;18:524–8.
[5] Koppe MJ, Zoetmulder FA, van Zandwijk N, Hart AA, Baas P, Rutgers EJ. The prognostic significance of a previous malignancy in operable non-small cell lung cancer. Lung Cancer 2001;32:47–53.
[6] Duchateau CS, Stokkel MP. Second primary tumors involving non-small cell lung cancer: prevalence and its influence on survival. Chest 2005;127: 1152–8.
[7] Hofmann HS, Neef H, Schmidt P. Primary lung cancer and extrapulmonary malignancy. Eur J Cardiothorac Surg 2007;32:653–8.
[8] Liu YY, Chen YM, Yen SH, Tsai CM, Perng RP. Multiple primary malignancies involving lung cancer-clinical characteristics and prognosis. Lung Cancer 2002;35:189–94.
[9] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69–90.
[10] Guggenheim DE, Shah MA. Gastric cancer epidemiology and risk factors. J Surg Oncol 2013;107:230–6.
[11] Ikeda Y, Saku M, Kawanaka H, Nonaka M, Yoshida K. Features of second primary cancer in patients with gastric cancer. Oncology 2003;65:113–7.
[12] Postmus PE, Brambilla E, Chansky K, Crowley J, Goldstraw P, Patz EF Jr *et al.* The IASLC Lung Cancer Staging Project: proposals for revision of the M descriptors in the forthcoming (seventh) edition of the TNM classification of lung cancer. J Thorac Oncol 2007;2:686–93.
[13] Furak J, Trojan I, Szoke T, Wolfard A, Nagy E, Nemeth I *et al.* Lung cancer as a second primary malignant tumor: prognostic values after surgical resection. Ann Thorac Surg 2008;7:50–3.
[14] Rice D, Kim HW, Sabichi A, Lippman S, Lee JJ, Williams B *et al.* The risk of second primary tumors after resection of stage I nonsmall cell lung cancer. Ann Thorac Surg 2003;76:1001–7; discussion 7–8.
[15] Maehara Y, Tomisaki S, Emi Y, Sakaguchi Y, Kusumoto T, Ichiyoshi Y *et al.* Clinicopathological features of patients who died with second primary cancer after curative resection for gastric cancer. Anticancer Res 1995;15: 1049–53.
[16] Ajiki W, Kinoshita N, Tsukuma H, Oshima A. Cancer incidence and incidence rates in Japan in 1996: estimates based on data from 10 population-based cancer registries. Jpn J Clin Oncol 2001;31:410–4.
[17] Chao A, Thun MJ, Henley SJ, Jacobs EJ, McCullough ML, Calle EE. Cigarette smoking, use of other tobacco products and stomach cancer mortality in US adults: the Cancer Prevention Study II. Int J Cancer 2002; 101:380–9.
[18] Johnson BE, Cortazar P, Chute JP. Second lung cancers in patients successfully treated for lung cancer. Semin Oncol 1997;24:492–9.
[19] Kinoshita Y, Tsukuma H, Ajiki W, Kinoshita N, Oshima A, Hiratsuka M *et al.* The risk for second primaries in gastric cancer patients: adjuvant therapy and habitual smoking and drinking. J Epidemiol 2000;10:300–4.

Cancer Science
Japanese Cancer Association JCA
Open Access

Immunophenotypic features of metastatic lymph node tumors to predict recurrence in N2 lung squamous cell carcinoma

Rie Matsuwaki,[1,2,3] Genichiro Ishii,[1] Yoshitaka Zenke,[1] Shinya Neri,[1] Keiju Aokage,[2] Tomoyuki Hishida,[2] Junji Yoshida,[2] Satoshi Fujii,[1] Haruhiko Kondo,[3] Tomoyuki Goya,[3] Kanji Nagai[2] and Atsushi Ochiai[1]

[1]Division of Pathology, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa; [2]Division of Thoracic Surgery, National Cancer Center Hospital East, Kashiwa; [3]Division of Thoracic Surgery, Kyorin University, Tokyo, Japan

Key words

Cancer-associated fibroblasts, lung squamous cell carcinoma, metastatic lymph node tumors, recurrence, tumor microenvironment

Correspondence

Genichiro Ishii and Atsushi Ochiai, Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba 277-0882, Japan.

Tel: 81-4-7133-1111; Fax: 81-4-7134-6865;
E-mails: gishii@east.ncc.go.jp and aochiai@east.ncc.go.jp

Funding Information

National Cancer Center; Foundation for the Promotion of Cancer Research; National Institute of Biomedical Innovation; Japan Society for the Promotion of Science (Kakenhi).

Received January 23, 2014; Revised April 22, 2014; Accepted April 25, 2014

Cancer Sci **105** (2014) 905–911

doi: 10.1111/cas.12434

Patients with mediastinal lymph node metastasis (N2) in squamous cell carcinoma (SqCC) of the lung have poor prognosis after surgical resection of the primary tumor. The aim of this study was to clarify predictive factors of the recurrence of pathological lung SqCC with N2 focusing on the biological characteristics of both cancer cells and cancer-associated fibroblasts (CAFs) in primary and metastatic lymph node tumors. We selected 64 patients with pathological primary lung N2 SqCC who underwent surgical complete resection and investigated the expressions of four epithelial–mesenchymal transition-related markers (caveolin, clusterin, E-cadherin, ZEB2), three cancer stem cell-related markers (ALDH-1, CD44 variant6, podoplanin) of cancer cells, and four markers of CAFs (caveolin, CD90, clusterin, podoplanin) in both primary and matched metastatic lymph node tumors in the N2 area. In the primary tumors, the expressions of all the examined molecules were not related to recurrence. However, in the metastatic lymph node tumors, high clusterin and ZEB2 expressions in the cancer cells and high podoplanin expression in the CAFs were significantly correlated with recurrence ($P = 0.03$, 0.04, and 0.007, respectively). In a multivariate analysis, only podoplanin expression in the CAFs in metastatic lymph node tumors was identified as a significantly independent predictive factor of recurrence ($P = 0.03$). Our study indicated that the immunophenotypes of both cancer cells and CAFs in metastatic lymph node tumors, but not primary tumors, provide useful information for predicting the recurrence of pathological N2 lung SqCC.

Many studies on predictive factors of recurrence have been carried out in NSCLC of various pathological stages. In particular, the pathological N factor, especially mediastinal lymph node metastasis (N2), has been considered an important predictor of recurrence.[1] The risk of distant metastasis and recurrence in patients with N2 in NSCLC is extremely high, and patients with N2 have a poor prognosis. The 5-year survival rate for pathological N2 NSCLC is reportedly 33.4%.[2]

Adenocarcinoma is the most common type of NSCLC, and a number of articles have discussed predictive factors of recurrence and prognosis. Squamous cell carcinoma is the second most common type, and the prognosis of patients with SqCC is more unfavorable than that for patients with adenocarcinoma because few anticancer drugs are available for treatment and the effects of these drugs are insufficient if the patients develop recurrence after surgery.[3,4] Moreover, information about predictive factors of recurrence is very limited. For this reason, the clinicopathological factors influencing recurrence in SqCC, particularly in the pathological N2 group which has a high risk of recurrence, need to be investigated.

Cancer tissue is composed of not only cancer cells, but also different kinds of stromal cells that are known as CAFs, tumor-associated macrophages, and immunoregulatory cells. The malignancy of cancer is not defined only by cancer cells. Biological analyses of non-cancer cells surrounding the cancer cells are also required, and their importance has been supported by many articles in recent years.[5,6]

To gain insight into the mechanism of cancer progression, the microenvironment of cancer at metastatic sites, in addition to primary sites, needs to be understood to determine the molecular mechanisms of cancer progression. At metastatic sites as well, cancer tissue is composed of not only cancer cells, but also the surrounding CAFs and other stromal cells such as lymphocytes and monocytes/macrophages. We previously reported that the presence of podoplanin-positive CAFs in metastatic lymph nodes, but not in primary tumors, predicted poor prognosis in pathological N2 stage III lung adenocarcinoma, suggesting that the biological characteristics of the cancer tissue in the metastatic lymph nodes may be more predictive of recurrence than that in the primary cancer tissue.[7]

The aim of this study was to identify how the immunophenotypic features of cancer cells and infiltrating CAFs in primary tumors and metastatic lymph node tumors could be correlated with recurrence for patients with pathological N2 SqCC. As for cancer cells, we focused on the cancer-initiating cell/cancer

stem cell and EMT-related molecules. In addition, we investigated the presence of CAFs with a tumor-promoting phenotype.

Materials and Methods

Subjects. A total of 546 consecutive patients with primary lung SqCC underwent surgical complete resection between July 1992 and December 2009 at the National Cancer Center Hospital East (Chiba, Japan). We excluded patients who did not undergo a standard operation or who had other cancers from the analyses. The number of pathological N0, N1, and N2 cases was 357 (65.4%), 125 (22.9%), and 64 (11.7%), respectively. The 3-year recurrence-free survival (RFS) rate and the 3-year overall survival rate of each group were significantly different ($P < 0.01$) (Table S1). Sixty-four cases with pathological N2 disease were enrolled in this study, and the median follow-up time was 5.3 years. The study was approved by the Ethics Committee of our institution.

Histological studies. The surgical specimens were fixed in 10% formalin or 100% methyl alcohol and embedded in paraffin. The tumors were cut into 5–10-mm thick slices, and serial 4-μm sections were stained using H&E. We counted the number of metastatic lymph nodes in the N2 area and measured the area of maximum metastatic lymph node tumors under a light microscope.

Immunofluorescence staining. Immunostaining was carried out using 4-μm paraffin-embedded tissue serial sections. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3% hydrogen peroxide in 100% methyl alcohol. After epitope retrieval, the slides were incubated with mouse anti-AE1/3 antibody (Leica Biosystems, Newcastle Upon Tyne, UK) for cancer cells and rabbit polyclonal anti-α-SMA antibody (Lab Vision, Fremont, CA, USA) for CAFs. Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) were used as the secondary antibody. Before mounting, all the sections were stained with DRAQ5TM (Alexis Biochemical, Lausen, Switzerland) to identify nucleated cells. After mounting, the fluorescent signals were analyzed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Antibodies and immunohistochemical staining. Information regarding the antibodies used in this study is shown in Table S2. Caveolin (clone D46G3; Cell Signaling, Danvers, MA, USA),[8,9] clusterin (clone 1A11; Acris Antibodies, Herford, Germany),[10,11] E-cadherin (clone 36; BD Biosciences, San Jose, CA, USA),[12,13] and ZEB2 (Novus Biologicals, Littleton, CO, USA)[14,15] were used as EMT-related markers. To evaluate the expression of cancer stem cell-related molecules, we used ALDH-1 (clone 44/ALDH; BD Biosciences),[16,17] CD44 variant 6 (clone VFF-7; Acris Antibodies),[18] and podoplanin (clone D2-40; Signet Antibodies, Princeton, NJ, USA).[19–22] To evaluate tumor-promoting CAFs, we used caveolin,[23] clusterin,[24] CD90 (Atlas Antibodies, Stockholm, Sweden),[25] and podoplanin.[5,7,26–28] After epitope retrieval, immunohistochemical staining was carried out as previously reported.[5–7]

Immunohistochemical scoring. All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (R.M. and G.I.) who had no knowledge of the patients' clinicopathological data. The labeling scores for cancer cells were calculated by multiplying the percentage of positive cancer cells per lesion (0–100%) by the staining intensity level (0, negative; 1, weak; 2, strong). Staining intensity 2 (strong) was defined as intensity level equal to positive control. Staining intensity 1 (weak) was defined as intermediate staining. We selected the median score to define high and low staining. A high staining score was defined as a score above the median value; a low score was defined as a score below the median value.

Cancer-associated fibroblasts were defined as stromal spindle cells that were morphologically identified as fibroblasts. As for the CAFs, cases with positive-stained spindle-shaped cells accounting for more than 10% of the cells in the cancer stroma were identified as the high expression group.

Statistical analysis. Recurrence-free survival was defined as the time from surgery until the time of the tumor recurrence or the date of the last follow-up. The survival curves were estimated using the Kaplan–Meier method, and the differences in survival between the subgroups were compared using the log–rank test. A multivariate analysis was carried out using the Cox proportional hazard model. The significance level was set at $P < 0.05$. Statistical analysis software (Stat View, version 5.0, SAS Institute Inc., Cary, NC, USA) was used to carry out the analyses.

Results

Patient characteristics and pathological factors of primary tumors. Univariate analyses of the clinical factors and the pathological factors in the primary tumors were carried out. A higher smoking index (>1000) was significantly correlated with a shorter interval until recurrence (Table 1). The other pathological factors were unrelated to recurrence.

Pathological factors of metastatic lymph node tumors. We carried out univariate analyses of pathological factors, the number of metastatic lymph nodes, and the station of N2. In addition, we measured the area of the metastatic lymph node tumor under a light microscope and univariate analysis was carried out (Table 2). However, the differences were not significant.

Cancer-associated fibroblasts in metastatic lymph node tumors. We confirmed that spindle cells had infiltrated the area around the cancer cells in the metastatic lymph node tumors, similar to the situation for the primary tumors (Fig. 1a,b). Double immunofluorescence staining revealed that the cancer cells were positive for AE1/3 (green) and that the spindle cells

Table 1. Univariate analysis of clinicopathological factors for recurrence-free survival (RFS) in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64)

Factor	No.	3-Year RFS, %	*P*-value
Gender			
Male/female	60/4	35.9/50.0	0.70
Age, years			
<65/≥65	29/35	40.6/33.2	0.68
Smoking index			
<1000/≥1000	39/25	49.2/22.2	0.01†
Pathological T status			
T1/T2–T4	42/22	40.0/33.9	0.71
Vascular invasion			
v(−)/v(+)	11/53	40.0/33.5	0.37
Lymphatic pemeation			
ly(−)/ly(+)	35/29	47.2/29.6	0.58
Pleural invasion			
pl(−)/pl(+)	28/36	42.3/30.6	0.25

†Significance.

Table 2. Univariate analysis of pathological factors in metastatic lymph node tumors for recurrence-free survival (RFS) in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64)

Factor	No.	3-Year RFS, %	P-value
No. of metastatic lymph nodes of N2 area			
1/>1	10/54	48.1/30.3	0.22
Station of N2			
Single/multiple	46/18	41.6/32.4	0.62
Area of the metastatic foci, mm^2			
<84/≥84	31/33	31.9/28.9	0.46

were positive for α-SMA (red), indicating that these cells were myofibroblasts (Fig. 1c,d). From these results, we confirmed that CAFs had also infiltrated the metastatic lymph node tumors, similar to the results of our previous study.[7]

Correlation between immunohistochemical staining of cancer cells and CAFs in primary tumors and prognostic impact. As for the cancer cells, we evaluated the expressions of ALDH-1, caveolin, CD44 variant 6, clusterin, E-cadherin, podoplanin, and ZEB2 (Table 3). In addition, the expressions of caveolin, CD90, clusterin, and podoplanin were analyzed in the CAFs. None of the expressions of any of the examined molecules in the primary tumors were related to recurrence.

Correlation between immunohistochemical staining of cancer cells and CAFs in metastatic lymph node tumors and prognostic impact. We carried out univariate analyses in the metastatic lymph node tumors (Table 4). A high clusterin expression level in cancer cells was observed in 24 cases (38%) (Fig. 2a,b). The 3-year RFS rate of cases with a high clusterin expression level was 28.6%, whereas that of cases with a low clusterin expression level was 45.2%. The difference between the two groups was significant (P = 0.04; Fig. 3a).

A high ZEB2 expression level in cancer cells was observed in 16 cases (25%) (Fig. 2c,d). Figure 3(b) shows the Kaplan–Meier curve for RFS in patients with pathological N2 SqCC according to the expression status of ZEB2 in the cancer cells. The 3-year RFS rate of cases with a high ZEB2 expression level was 15.6%, while that of cases with a low ZEB2 expression level was 46.3%. High ZEB2 expression in cancer cells in metastatic lymph node tumors was significantly correlated with a shorter interval until recurrence, compared with low ZEB2 expression in the cancer cells (P = 0.03; Fig. 3b).

A high podoplanin expression level in the CAFs was observed in 27 cases (42%) (Fig. 2e,f). The 3-year RFS rate of cases with a high podoplanin expression level was 19.8%, while that of cases with a low podoplanin expression level was

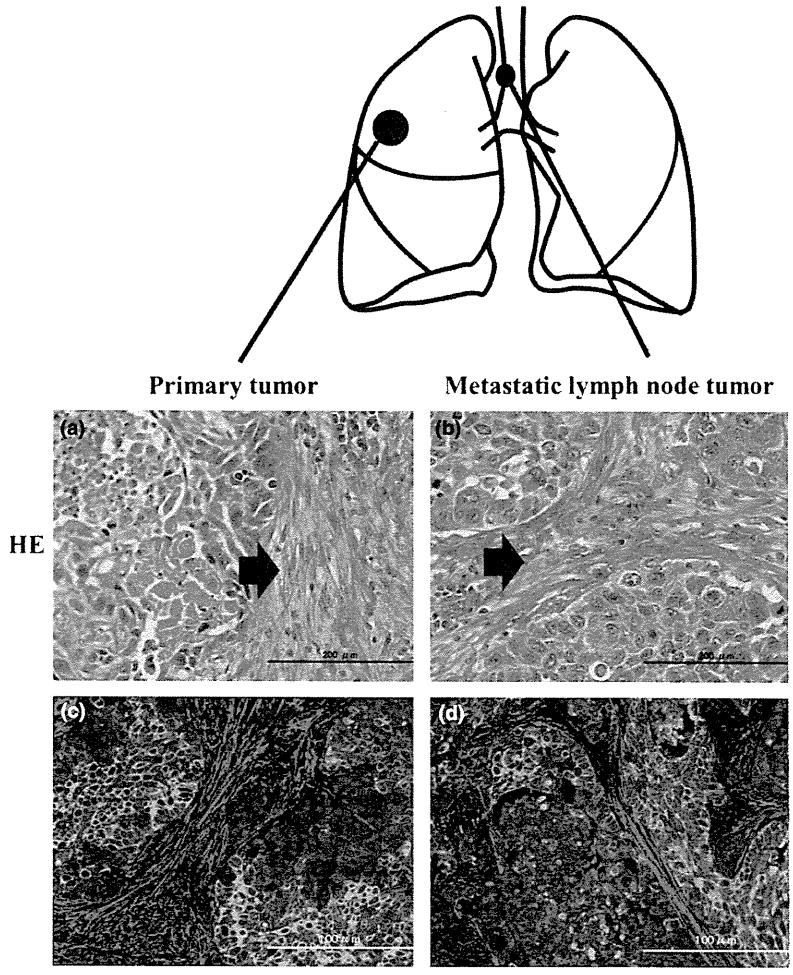

Fig. 1. Staining with H&E and double immunofluorescence staining of tumor cells in a primary and a metastatic lymph node tumor in a patient with squamous cell carcinoma of the lung. (a, b) H&E staining in the primary tumor (a) and the metastatic lymph node tumor (b). Arrows indicate cancer-associated fibroblasts (CAFs). (c) Double immunofluorescence staining in the primary tumor. Blue, nucleus; green, AE1/3-positive cancer cells; red, α-smooth muscle actin-positive myofibroblasts (CAFs). (d) Double immunofluorescence staining in the metastatic lymph node tumor. Blue, nucleus; green, AE1/3-positive cancer cells; red, α-smooth muscle actin-positive myofibroblasts (CAFs).

Table 3. Univariate analysis of immunochemical staining of (a) cancer cells and (b) cancer-associated fibroblasts in primary tumors in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64)

Antibodies	Median score	High	Low	3-Year RFS, %	*P*-value
(a)					
EMT-related molecules					
Caveolin	0	29	34	High, 47.2 Low, 32.0	0.34
Clusterin	10	34	30	High, 33.3 Low, 45.2	0.12
E-cadherin	48	32	32	High, 39.9 Low, 34.4	0.87
ZEB2	0	33	31	High, 40.1 Low, 36.1	0.79
Stem cell-related molecules					
ALDH-1	123	32	32	High, 32.3 Low, 45.1	0.21
CD44 variant 6	65	32	32	High, 32.5 Low, 41.4	0.60
Podoplanin	10	30	34	High, 48.0 Low, 28.9	0.15

Antibodies	High	Low	3-Year RFS, %	*P*-value
(b)				
Cancer-associated fibroblasts				
Caveolin	29	35	High, 35.6 Low, 39.3	0.98
CD90	55	9	High, 35.8 Low, 44.4	0.23
Clusterin	45	18	High, 38.8 Low, 31.0	0.88
Podoplanin	47	17	High, 33.8 Low, 52.3	0.12

EMT, epithelial–mesenchymal transition; RFS, recurrence-free survival; RFS, recurrence-free survival.

Table 4. (a) Univariate analysis of immunochemical staining of cancer cells in metastatic tumors in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64); (b) Univariate analysis of immunochemical staining of cancer-associated fibroblasts in metastatic lymph node tumors in patients with resected pathological N2 squamous cell carcinoma of the lung

Antibodies	Median score	High	Low	3-Year RFS, %	*P*-value
(a)					
EMT-related molecules					
Caveolin	0	21	43	High, 45.4 Low, 32.8	0.60
Clusterin	0	24	40	High, 28.6 Low, 45.2	0.04†
E-cadherin	30	31	33	High, 44.7 Low, 32.7	0.30
ZEB2	0	16	48	High, 15.6 Low, 46.3	0.03†
Stem cell-related molecules					
ALDH-1	128	32	32	High, 34.4 Low, 45.1	0.20
CD44 variant 6	30	33	31	High, 32.3 Low, 42.3	0.35
Podoplanin	0	17	47	High, 43.0 Low, 34.7	0.60

Antibodies	High	Low	3-Year RFS,%	*P*-value
(b)				
Cancer-associated fibroblasts				
Caveolin	3	61	High, 33.3 Low, 39.2	0.750
CD90	32	32	High, 37.5 Low, 39.1	0.260
Clusterin	24	40	High, 28.7 Low, 43.7	0.210
Podoplanin	27	37	High, 19.8 Low, 52.6	0.007†

†Significance. EMT, epithelial–mesenchymal transition; RFS, recurrence-free survival; RFS, recurrence-free survival.

52.6%. High podoplanin expression in the CAFs in metastatic lymph node tumors was significantly correlated with a shorter interval until recurrence, compared with low podoplanin expression in the CAFs (P = 0.007, Fig. 3c).

The expressions of clusterin and ZEB2 in cancer cells and the expression of podoplanin in CAFs in metastatic lymph node tumors were significantly correlated with those in the primary tumors (Table S3).

Multivariate analyses to identify factors significantly associated with recurrence. A multivariate analysis using the Cox proportional hazard model was carried out to determine the recurrence of conventional clinicopathological factors (Table 5). Only podoplanin expression in CAFs in metastatic lymph node tumors was identified as a significantly independent predictor of RFS (P = 0.03).

Discussion

This is the first report to discuss the prognostic importance of the tumor microenvironment of metastatic lymph node tumors. In this study, we identified clusterin and ZEB2 expression in cancer cells and podoplanin expression in CAFs in metastatic lymph node tumors as significant predictive factors of recurrence in patients with pathological N2 SqCC. However, none of the expression levels of the molecules examined in the primary tumors were significantly correlated with recurrence. Few studies to date have examined prognostic significance by considering the biological characteristics of both the primary tumors and the metastatic lymph node tumors in advanced-stage cases with lymph node metastasis.[29] Fukuse *et al.* reported that a high expression level of proliferating cell nuclear antigen in both the primary tumors and metastatic lymph node tumors was a significant predictor of a poor prognosis in pathological N2 NSCLC.[30] In addition, CAFs also reportedly exist in metastatic lymph node tumors, and the EMT is influenced by CAFs.[31,32] We previously reported that the presence of podoplanin-positive CAFs in metastatic lymph node tumors, but not in primary tumors, predicted poor prognosis in patients with pathological N2 stage III lung adenocarcinoma.[7] Taken together, predictive factors of recurrence in patients with lymph node metastasis should be analyzed with due consideration given to the metastatic tumor microenvironment.

We previously reported that the presence of podoplanin-positive CAFs in primary tumor is correlated with poorer prognosis in stage I SqCC, which was inconsistent with the results of our current study.[28] This would also support the biological impor-

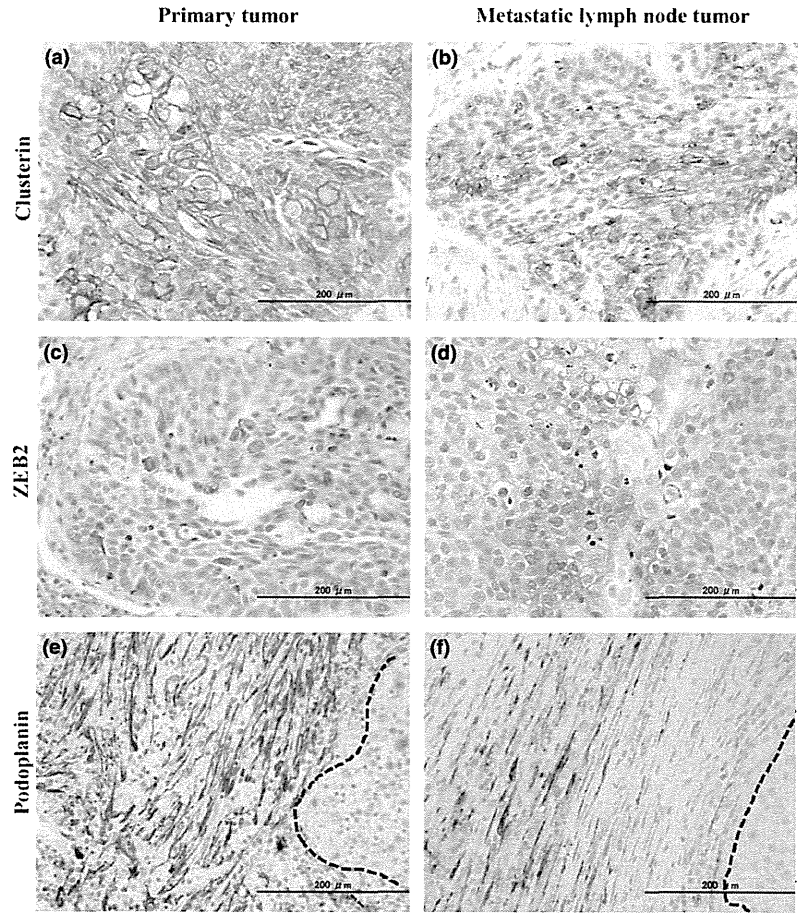

Fig. 2. Immunohistochemical staining of tumor cells in a primary tumor and a metastatic lymph node tumor in a patient with squamous cell carcinoma of the lung. (a,b) Clusterin expression of cancer cells in the primary tumor (a) and the metastatic lymph node tumor (b). (c,d) ZEB2 expression of cancer cells in the primary tumor (c) and the metastatic lymph node tumor (d). (e,f) Podoplanin expression of cancer-associated fibroblasts in the primary tumor (e) and the metastatic lymph node tumor (f). Dotted lines show the margin of the cancer cell nest.

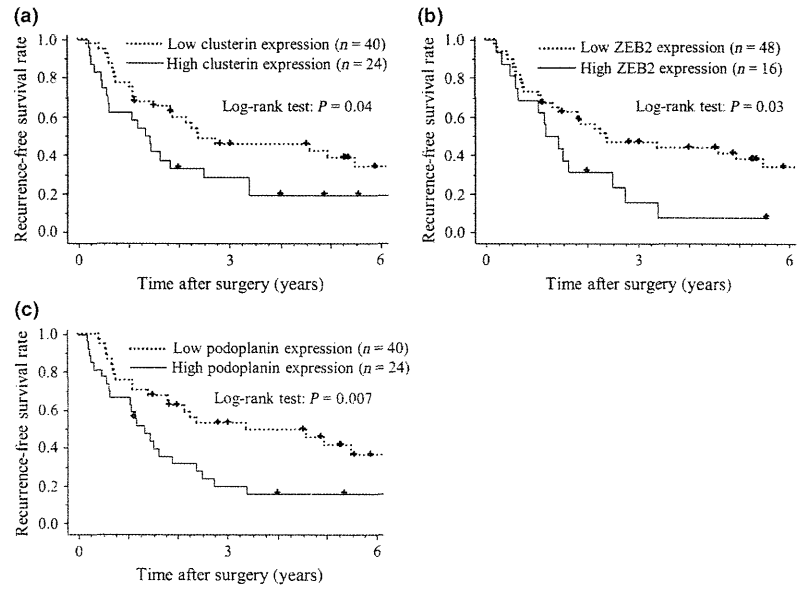

Fig. 3. Kaplan–Meier recurrence-free survival (RFS) curve for patients with resected pathological N2 squamous cell carcinoma of the lung according to immunohistochemical staining. (a) Kaplan–Meier RFS curve according to clusterin expression of cancer cells in metastatic lymph node tumors. (b) Kaplan–Meier RFS curve according to ZEB2 expression of cancer cells in metastatic lymph node tumors. (c) Kaplan–Meier RFS curve according to podoplanin expression of cancer-associated fibroblasts in metastatic lymph node tumors.

tance of cancer tissue in metastatic sites of advanced cancer (N2 disease).

Tumor metastasis has been postulated to start with EMTs, a process through which a small number of tumor cells at the primary site acquire a more invasive and metastatic phenotype. After engraftment at metastatic sites, tumor cells with subsequent mesenchymal–epithelial transitions, the reverse phenomenon of EMTs, develop metastatic tumors and recruit certain sorts of CAFs. Thus, the microenvironment of metastatic tumors created by cancer cells and surrounding CAFs might differ from that of the primary tumors. This difference could explain why the biological characteristics of metastatic lymph

Table 5. Multivariate analysis of clinicopathological factors for recurrence-free survival in patients with resected pathological N2 squamous cell carcinoma of the lung (n = 64)

Factor	Hazard ratio	(95% CI)	P-value
Smoking index			
≥1000/<1000	1.92	(0.83–2.92)	0.17
Clusterin expression of cancer cells in metastatic lymph node tumors			
High/low	1.55	(0.74–2.58)	0.30
ZEB2 expression of cancer cells in metastatic lymph node tumors			
High/low	1.39	(0.96–3.82)	0.06
Podoplanin expression of CAFs in metastatic lymph node tumors			
High/low	2.00	(1.08–3.72)	0.03†

†Significance. CAF, cancer-associated fibroblasts; CI, confidence interval.

node tumors were more strongly predictive of recurrence than those of the primary tumors.

Podoplanin is 40-kD glycoprotein for type I transmembrane sialomucin participating in platelet aggregation, invasion, and metastasis of cancer. Recent studies, including some by our group, have identified podoplanin as a marker of tumor-promoting CAFs in lung adenocarcinoma, SqCC, and breast cancer.[26–28,33] Our current study showed that the presence of podoplanin-positive CAFs in metastatic lymph node tumors, but not in primary tumors, participated in recurrence, similar to the results observed for adenocarcinoma with N2 disease.[7] The metastatic microenvironment created by both podoplanin-expressing CAFs and cancer cells may confer an additional malignant potential to metastasized cancer cells, such as effects on migration, proliferation, and survival. Moreover, podoplanin expression was the most significant predictor of RFS. Thus, consideration of the biological characteristics of CAFs in metastatic lymph node tumors might be very important for determining the likelihood of recurrence after surgery.

Clusterin, a stress-activated and apoptosis-associated molecular chaperone that confers survival and a proliferative advantage to cancer cells, is an important mediator of the transforming growth factor-β-induced EMT.[11] Clusterin overexpression in cancer cells upregulates metastasis and is related to chemoresistance.[10,34] ZEB2 is one of the transcription factors that regulates the expression of E-cadherin and mediates the EMT. ZEB2 overexpression in the cancer cells of primary tumors was reportedly correlated with a poor prognosis in several types of cancers.[14,35] Kurahara *et al.*[15] reported that pancreatic cancer cells in metastatic lymph node tumors expressed high levels of ZEB1 and ZEB2, suggesting that these cancer cells were associated with the EMT phenotype. In the current study, high expression levels of the EMT-related markers, clusterin and ZEB2 in cancer cells at metastatic lymph node tumors were significantly correlated with a shorter time until recurrence. These findings suggest that the EMT phenotypes of cancer cells that have detached from the primary tumors are likely to be an important determinant of the development of remote metastasis.

The conversion to the EMT phenotype of cancer cells is mediated by several factors, and E-cadherin is known to be an EMT-related marker. In this study, a low E-cadherin expression level in cancer cells at metastatic lymph node tumors was not correlated with recurrence. No inverse correlations between clusterin or ZEB2 expression and E-cadherin expression in metastatic lymph node cancer cells was seen (data not shown). This discrepancy may be explained by the fact that the expression of E-cadherin is regulated not only by numerous EMT-related transcription factors such as ZEB1, ZEB2, Twist, and Snail, but also by epigenetic mechanisms.

In conclusion, we found that clusterin and ZEB2 expression in cancer cells and podoplanin expression in CAFs in metastatic lymph node tumors were significant predictive factors of cancer recurrence. The prognostic importance of the microenvironment in primary tumors has already been reported for early-stage cases, but the current study also suggests the need to examine the microenvironment in metastatic lymph node tumors in advanced-stage cases. Although a prospective study with a larger number of patients and a multicenter study are warranted, this study has important implications for investigations focusing on the microenvironment in metastatic lymph node tumors, and should provide a significant indicator to future directionality.

Acknowledgments

This work was supported by the National Cancer Center Research and Development Fund (23-A-12 and 23-K-18), the Foundation for the Promotion of Cancer Research, Third-Term Comprehensive 10-Year Strategy for Cancer Control, the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation, and the Japan Society for the Promotion of Science Kakenhi program (24659185).

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

CAFs	cancer-associated fibroblasts
EMT	epithelial–mesenchymal transition
MET	mesenchymal–epithelial transition
NSCLC	non-small-cell lung cancer
RFS	recurrence-free survival
SMA	smooth muscle actin
SqCC	squamous cell carcinoma

References

1 Goldstraw P, Crowley J, Chansky K *et al.* The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumors. *J Thorac Oncol* 2007; **2**: 706–14.
2 Hanagiri T, Takenaka M, Oka S *et al.* Clinical significance in the number of involved lymph nodes in patients that underwent surgery for pathological stage III-N2 non-small cell lung cancer. *J Cardiothorac Surg* 2011; **6**: 144.
3 Sereno M, Esteban IR, Zambrana F *et al.* Squamous-cell carcinoma of the lungs: is it really so different? *Crit Rev Oncol Hematol* 2012; **84**: 327–39.
4 Fiala O, Pesek M, Finek J *et al.* Gene mutations in squamous cell NSCLC: insignificance of EGFR, KRAS and PIK3CA mutations in prediction of EGFR-TKI treatment efficacy. *Anticancer Res* 2013; **33**: 1705–11.
5 Ito M, Ishii G, Nagai K *et al.* Prognostic impact of cancer-associated stromal cells in patients with stage I lung adenocarcinoma. *Chest* 2012; **142**: 151–8.
6 Hirayama S, Ishii G, Nagai K *et al.* Prognostic impact of CD204-positive macrophages in lung squamous cell carcinoma: possible contribution of CD204-positive macrophages to the tumor-promoting microenvironment. *J Thorac Oncol* 2012; **7**: 1790–7.
7 Neri S, Ishii G, Taira T *et al.* Recruitment of podoplanin positive cancer-associated fibroblasts in metastatic lymph nodes predicts poor prognosis in

pathological N2 stage III lung adenocarcinoma. *Ann Surg Oncol* 2012; **19**: 3953–62.
8 Bailey KM, Liu J. Caveolin-1 up-regulation during epithelial to mesenchymal transition is mediated by focal adhesion kinase. *J Biol Chem* 2008; **283**: 13714–24.
9 Masuelli L, Budillon A, Marzocchella L *et al.* Caveolin-1 overexpression is associated with simultaneous abnormal expression of the E-cadherin/alpha-beta catenins complex and multiple ErbB receptors and with lymph nodes metastasis in head and neck squamous cell carcinomas. *J Cell Physiol* 2012; **227**: 3344–53.
10 Harada K, Miyake H, Kusuda Y *et al.* Expression of epithelial-mesenchymal transition markers in renal cell carcinoma: impact on prognostic outcomes in patients undergoing radical nephrectomy. *BJU Int* 2012; **110**: E1131–7.
11 Shiota M, Zardan A, Takeuchi A *et al.* Clusterin mediates TGF-beta-induced epithelial-mesenchymal transition and metastasis via Twist1 in prostate cancer cells. *Cancer Res* 2012; **72**: 5261–72.
12 Koay MH, Crook M, Stewart CJ. Cyclin D1, E-cadherin and beta-catenin expression in FIGO Stage IA cervical squamous carcinoma: diagnostic value and evidence for epithelial-mesenchymal transition. *Histopatholog* 2012; **61**: 1125–33.
13 Sakamoto K, Imanishi Y, Tomita T *et al.* Overexpression of SIP1 and down-regulation of E-cadherin predict delayed neck metastasis in stage I/II oral tongue squamous cell carcinoma after partial glossectomy. *Ann Surg Oncol* 2012; **19**: 612–9.
14 Cai MY, Luo RZ, Chen JW *et al.* Overexpression of ZEB2 in peritumoral liver tissue correlates with favorable survival after curative resection of hepatocellular carcinoma. *PLoS ONE* 2012; **7**: e32838.
15 Kurahara H, Takao S, Maemura K *et al.* Epithelial-mesenchymal transition and mesenchymal-epithelial transition via regulation of ZEB-1 and ZEB-2 expression in pancreatic cancer. *J Surg Oncol* 2012; **105**: 655–61.
16 Dimou A, Neumeister V, Agarwal S *et al.* Measurement of aldehyde dehydrogenase 1 expression defines a group with better prognosis in patients with non-small cell lung cancer. *Am J Pathol* 2012; **181**: 1436–42.
17 Okudela K, Woo T, Mitsui H *et al.* Expression of the potential cancer stem cell markers, CD133, CD44, ALDH1, and beta-catenin, in primary lung adenocarcinoma–their prognostic significance. *Pathol Int* 2012; **62**: 792–801.
18 Shi J, Zhou Z, Di W *et al.* Correlation of CD44v6 expression with ovarian cancer progression and recurrence. *BMC Cancer* 2013; **13**: 182.
19 Atsumi N, Ishii G, Kojima M *et al.* Podoplanin, a novel marker of tumor-initiating cells in human squamous cell carcinoma. *Biochem Biophys Res Commun* 2008; **373**: 36–41.
20 Shimada Y, Ishii G, Nagai K *et al.* Expression of podoplanin, CD44, and p63 in squamous cell carcinoma of the lung. *Cancer Sci* 2009; **100**: 2054–9.
21 Rahadiani N, Ikeda J, Makino T *et al.* Tumorigenic role of podoplanin in esophageal squamous-cell carcinoma. *Ann Surg Oncol* 2010; **17**: 1311–23.
22 Nishijima N, Ishii G, Nagai K *et al.* Cancer-initiating cell marker-positive cells generate metastatic tumors that recapitulate the histology of the primary tumors. *Pathol Int* 2013; **63**: 94–101.
23 Goetz JG, Minguet S, Navarro-Lerida I *et al.* Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell* 2011; **146**: 148–63.
24 Niu ZH, Wang Y, Chun B *et al.* Secretory clusterin (sCLU) overexpression is associated with resistance to preoperative neoadjuvant chemotherapy in primary breast cancer. *Eur Rev Med Pharmacol Sci* 2013; **17**: 1337–44.
25 Tang KH, Dai YD, Tong M *et al.* A CD90(+) tumor-initiating cell population with an aggressive signature and metastatic capacity in esophageal cancer. *Cancer Res* 2013; **73**: 2322–32.
26 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**: 1053–9.
27 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.
28 Ono S, Ishii G, Nagai K *et al.* 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. *Chest* 2013; **143**: 963–70.
29 Mori T, Hirota T, Ohashi Y *et al.* Significance of histologic type of primary lesion and metastatic lymph nodes as a prognostic factor in stage III colon cancer. *Dis Colon Rectum* 2006; **49**: 982–92.
30 Fukuse T, Hirata T, Naiki H *et al.* Prognostic significance of proliferative activity in pN2 non-small-cell lung carcinomas and their mediastinal lymph node metastases. *Ann Surg* 2000; **232**: 112–8.
31 Vered M, Dayan D, Yahalom R *et al.* Cancer-associated fibroblasts and epithelial-mesenchymal transition in metastatic oral tongue squamous cell carcinoma. *Int J Cancer* 2010; **127**: 1356–62.
32 Yeung TM, Buskens C, Wang LM *et al.* Myofibroblast activation in colorectal cancer lymph node metastases. *Br J Cancer* 2013; **108**: 2106–15.
33 Schoppmann SF, Berghoff A, Dinhof C *et al.* Podoplanin-expressing cancer-associated fibroblasts are associated with poor prognosis in invasive breast cancer. *Breast Cancer Res Treat* 2012; **134**: 237–44.
34 Hassan MK, Watari H, Han Y *et al.* Clusterin is a potential molecular predictor for ovarian cancer patient's survival: targeting clusterin improves response to paclitaxel. *J Exp Clin Cancer Res* 2011; **30**: 113.
35 Fang Y, Wei J, Cao J *et al.* Protein expression of ZEB2 in renal cell carcinoma and its prognostic significance in patient survival. *PLoS ONE* 2013; **8**: e62558.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Overall survival and recurrence-free survival in 546 patients with resected squamous cell carcinoma.

Table S2. Antibodies used in the immunohistochemical staining.

Table S3a. Correlation of clusterin expression between cancer cells in primary tumors and metastatic lymph node tumors.

Table S3b. Correlation of ZEB2 expression between cancer cells in primary tumors and metastatic lymph node tumors.

Table S3c. Correlation of podoplanin expression between cancer-associated fibroblasts in primary tumors and metastatic lymph node tumors.

Prognostic Impact of Microscopic Vessel Invasion and Visceral Pleural Invasion in Non–Small Cell Lung Cancer

A Retrospective Analysis of 2657 Patients

Shinya Neri, MD, Junji Yoshida, MD, PhD,* Genichiro Ishii, MD, PhD,† Yuki Matsumura, MD,* Keiju Aokage, MD, PhD,* Tomoyuki Hishida, MD, PhD,* and Kanji Nagai, MD, PhD**

Objective: We aimed to assess the prognostic significance of microscopic vessel invasion (MVI) and visceral pleural invasion (VPI) in non–small cell lung cancer (NSCLC).

Background: VPI is included in the current tumor-node-metastasis (TNM) classification in NSCLC; however, MVI is not incorporated in TNM classification.

Methods: From August 1992 to December 2009, 2657 consecutive patients with pathological T1-4N0-2M0 NSCLC underwent complete resection. In addition to conventional staging factors, we evaluated MVI histologically and analyzed its significance in NSCLC recurrence prognosis. The recurrence-free period in several NSCLC subgroups was analyzed using the Kaplan-Meier method and Cox regression analysis.

Results: The proportion of patients with a 5-year recurrence-free period was 52.6% and 87.5%, respectively, in those with and without MVI ($P < 0.001$). Multivariate analysis showed that MVI, similarly to VPI, was found to be an independently significant predictor of recurrence [hazard ratio (HR): 2.78]. In particular, MVI and VPI were the 2 strongest significant independent predictors of recurrence in 1601 patients with pathological stage I disease treated without adjuvant chemotherapy (HR: 2.74 and 1.84, respectively). In each T subgroup analysis, evident and significant separation of the recurrence-free proportion curves were observed among the 3 groups (VPI and MVI absent, VPI or MVI present, and VPI and MVI present).

Conclusions: This study demonstrated that MVI was a significant independent risk factor for recurrence in patients with a resected T1-4N0-2M0 NSCLC. Further data on MVI prognostic impact should be collected for the next revision of the TNM staging system.

Keywords: non–small cell lung cancer, pleural invasion, vessel invasion

(*Ann Surg* 2014;260:383–388)

Visceral pleural invasion (VPI), a pathological invasive finding, has been reported to be a poor prognostic factor in patients with non–small cell lung cancer (NSCLC).[1–4] Therefore, VPI is incorporated in the current seventh edition of the Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) classification.[5] Recent studies have demonstrated microscopic vessel invasion (MVI), including blood vessel invasion (BVI) or lymphatic vessel invasion (LVI), as a strong independent factor of poor prognosis in patients with NSCLC.[6–9] However, MVI has not been incorporated in TNM classification.

In this study, we assessed the prognostic significance of MVI in a large number of patients with pathological T1-4N0-2M0 NSCLC who were treated in our institution and were investigated to elucidate whether or not MVI should be incorporated in the future TNM classification.

METHODS

Patients

From August 1992 to December 2009, 2657 consecutive patients with pathological T1-4N0-2M0 NSCLC underwent complete resection. We defined complete resection as a segmentectomy or greater with systematic ipsilateral hilar and mediastinal lymph node dissection. Patients who underwent induction chemotherapy or radiotherapy and patients with evidence of residual tumor at the surgical margin were excluded from the study. The tumors were staged according to the seventh edition of the UICC TNM classification[5] and were histologically subtyped and graded according to the World Health Organization guidelines.[10] Informed consent for the use and analyses of clinical data was obtained preoperatively from each patient. The institutional review board approved the study protocol. Collection and evaluation of the clinical data were performed similarly to our previous studies.[1,11–13]

Histopathological Studies

All surgical specimens were fixed in 10% formalin and embedded in paraffin. Serial 4-μm sections were stained with hematoxylin and eosin (H&E). BVI and VPI were evaluated by H&E and elastin (Victoria blue–van Gieson) stains. LVI was evaluated by H&E and, when necessary, by lymphatic endothelial (D2-40) stains. Blood vessels were identified by the presence of erythrocytes in the lumen or the presence of elastic tissue around larger vessels. Lymphatic vessels were identified by exclusion: lacking of erythrocytes or the presence of lymphatic endothelial layer or lymphocytes. The presence of BVI or LVI was determined by identifying conspicuous clusters of intravascular cancer cells surrounded by blood or lymphatic vessels, respectively (Figs. 1A, B, C, D). MVI was defined as the presence of BVI and/or LVI. Pathological evaluation was reviewed for consistency by a single pathologist (G.I.), and 1 or more pathologists confirmed the diagnoses. All these pathologists were unaware of the clinical data.

Patient Follow-up

We examined patients at outpatient clinics at 3-month intervals for the first 2 years and at 6-month intervals thereafter.

From the *Division of Thoracic Surgery, National Cancer Center Hospital East; and †Pathology Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan.

Disclosure: Junji Yoshida serves as a consultant to Johnson & Johnson Japan (ongoing) and has received a research grant from Konica Minolta Japan (current). He served as a consultant to Covidien Japan (past) and received a research grant from GlaxoSmithKline (past). The other authors declare no conflicts of interest.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.annalsofsurgery.com).

Reprints: Junji Yoshida, MD, PhD, Division of Thoracic Surgery, National Cancer Center Hospital East, 6-5-1, Kashiwanoha, Kashiwa, Chiba 277-8577, Japan. E-mail: jyoshida@east.ncc.co.jp.

ISSN: 0003-4932/14/26002-0383
DOI: 10.1097/SLA.0000000000000617

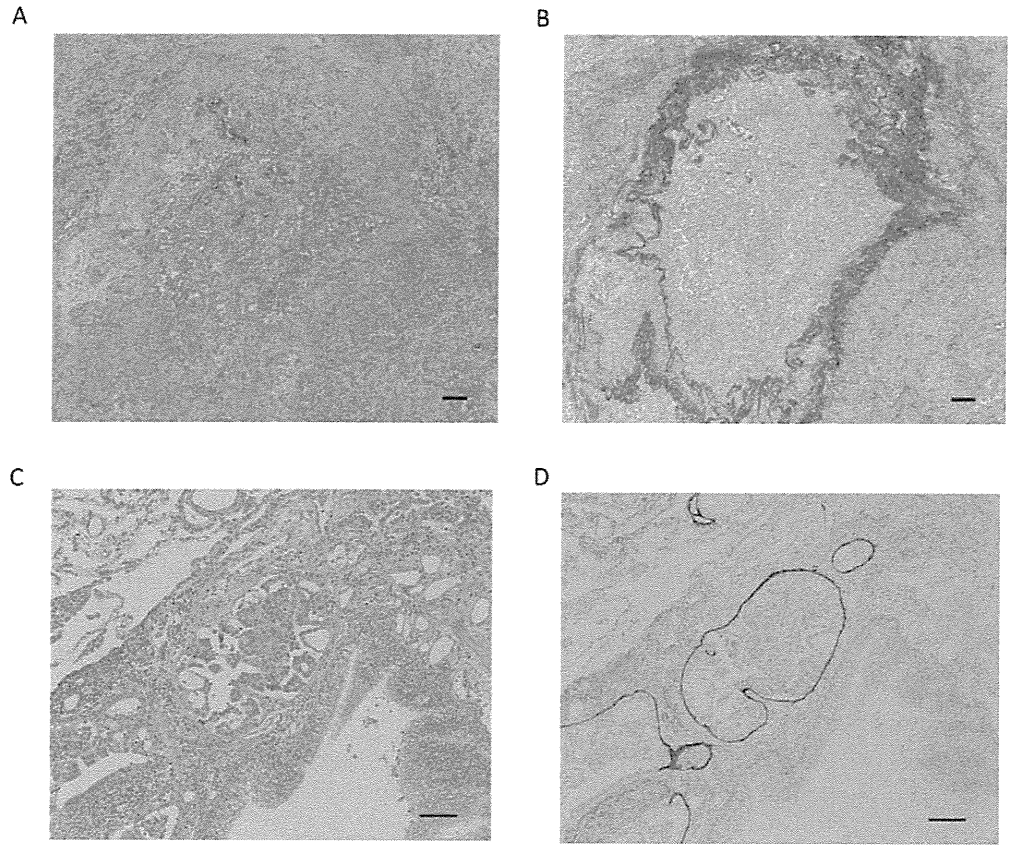

FIGURE 1. Microscopic findings of BVI (A and B) and LVI (C and D). A, BVI with H&E staining; B, BVI with Victoria blue–van Gieson staining; C, LVI with H&E staining; and D, LVI with D2-40 staining. Scale bar, 100 μm.

Follow-up evaluation included physical examination, chest radiography, and blood examination (including pertinent tumor markers). Further evaluations, including computed tomographic scans of the chest and abdomen, brain magnetic resonance imaging, and bone scintigraphy, were performed when symptoms or signs of recurrence were detected. We used integrated positron emission tomography and computed tomographic scan since 2004. Recurrent NSCLC was diagnosed by physical examination and diagnostic imaging of lesions consistent with recurrent lung cancer. Diagnosis was histologically confirmed when clinically feasible. The date of recurrence was defined as the date of histological proof or the date of identification by a physician in cases diagnosed on the basis of clinicoradiological findings. In patients with recurrent lesions simultaneously diagnosed at multiple sites, the site of main clinical interest was recorded.

Evaluation of Clinicopathological Factors

Clinical characteristics were retrieved from the clinical records and our division database: age (dichotomized at the median age of 66 years), sex, smoking habits (non- or ever smoker), preoperative serum level of carcinoembryonic antigen (CEA; cutoff at the normal upper limit of 5 ng/mL), and pattern of failure. As described in the section on histopathological studies, the following factors were reviewed and confirmed by the pathologists: diameter of the tumor in the resected specimens ($\leq$3 cm or >3 cm), histological type (adenocarcinoma or nonadenocarcinoma), VPI (presence or absence defined using the 7th edition of TNM classification), MVI (presence or absence), and lymph node metastasis (N0, N1, or N2, as defined using the seventh edition of TNM classification).

Statistical Analyses

Correlations between categorical outcomes were evaluated using the Fisher exact test. The overall survival time was measured from the date of surgery to the date of death from any cause or last follow-up. The length of the recurrence-free period was measured from the date of surgery to the date of the first recurrence or last follow-up. For the analysis of recurrence-free proportion, patients who died without recurrence or who were known to have no recurrence at the date of last contact were censored. Cumulative survival rates or recurrence-free proportions were calculated by the Kaplan-Meier method, and the differences in survival curves were compared using the log-rank test. The Cox proportional hazard model was used in the univariate and multivariate analyses of recurrence-free proportion. The significance level was set at $P < 0.05$. All statistical analyses were performed using the statistical softwares SPSS 11.0 (SPSS Inc, Chicago, IL) and JMP 9 (SAS Institute, Cary, NC).

RESULTS

Table 1 summarizes the patient characteristics. A total of 2657 patients [1697 men (63.9%) and 960 women (36.1%)] aged between 29 and 89 years (median, 66 years) were enrolled in the study. More than 90% of the patients underwent lobectomy, including bilobectomy. Adenocarcinoma was the most common histological finding (66.4%). The median follow-up period was 5.0 years. The 5-year overall survival rate for patients with MVI was 57.2%, which was significantly lower than that for patients without MVI (87.3%; $P < 0.001$). Of the 2657 patients, 822 (30.9%) developed recurrence. Recurrence was locoregional only in 344 patients and distant in 478

TABLE 1. Clinicopathological Characteristics of All Stage Patients With T1-4N0-2M0 NSCLC

Variable	n (%)
Age, yrs	
Median = 66 (range: 22–89)	
≤65	1284 (48.7)
>65	1354 (51.3)
Sex	
Female	960 (36.1)
Male	1697 (63.9)
Smoking history	
Never smokers	838 (31.6)
Ever smokers	1800 (67.7)
NA	19 (0.7)
CEA, ng/mL	
≤5	1697 (63.9)
>5	945 (35.6)
NA	15 (0.6)
Surgery	
Segmentectomy	31 (1.2)
Lobectomy	2497 (94.0)
Pneumonectomy	129 (4.9)
Histology	
Adenocarcinoma	1764 (66.4)
Squamous cell carcinoma	605 (22.8)
Large cell carcinoma	145 (5.5)
Adenosquamous carcinoma	66 (2.5)
Others	77 (2.9)
Tumor size, cm	
≤3	1478 (55.6)
>3	1179 (44.4)
pN status	
pN0	1958 (73.7)
pN1	390 (14.7)
pN2	309 (11.6)
VPI	
Absent	1769 (66.6)
Present	888 (33.4)
MVI	
Absent	1186 (44.6)
Present	1471 (55.4)

NA indicates not available.

TABLE 2. Initial Site of Failure in Patients With Recurrence

	No. Patients	No. Recurrences	Initial site of recurrence: Locoregional Only (%)	Initial site of recurrence: Distant (%)	P*
Overall	2657	822	344 (41.8)	478 (58.2)	
MVI					
Absent	1186	149	80 (53.7)	69 (46.3)	0.001
Present	1471	673	264 (39.2)	409 (60.8)	

*Fisher exact test.

patients. Of the patients who developed recurrence, distant metastases were found more frequently in patients with MVI ($P < 0.001$; Table 2). The proportion of patients with MVI who had a 5-year recurrence-free period was 52.6%, which was significantly lower than that for patients without MVI (87.5%; $P < 0.001$; Fig. 2).

Univariate analysis using the Cox regression model showed that age, sex, smoking history, preoperative serum CEA level, histological type, tumor size, pathological nodal (pN) status, VPI, and MVI were significantly associated with recurrence (Table 3). Multivariate analysis showed that age, preoperative serum CEA level, histological type, tumor size, pN status, VPI, and MVI were significant independent predictors of recurrence (Table 3).

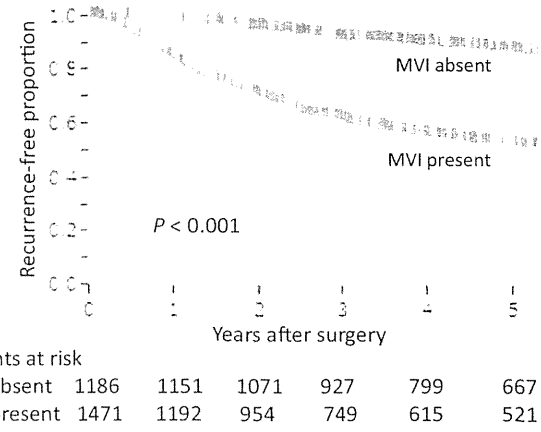

FIGURE 2. Recurrence-free proportion curves of patients with or without MVI.

Table 4 shows the characteristics of the 1601 patients with pathological stage I disease who did not receive cytotoxic adjuvant chemotherapy. Clinicopathological risk factors for recurrence were analyzed in these patients. Univariate analysis revealed that age, sex, smoking history, preoperative serum CEA level, histological type, tumor size, VPI, and MVI were significantly associated with recurrence, whereas multivariate analysis showed that age, smoking history, VPI, and MVI were significant independent predictors of recurrence (Table 5). The 2 strongest risk factors for recurrence were MVI and VPI with hazard ratios (HRs) of 2.74 and 1.84, respectively.

We divided the cases into 4 groups according to the status of VPI and MVI. In the 4 groups with or without VPI/MVI (−/−, +/−, −/+, and +/+), the proportion of patients with a 5-year recurrence-free period were 91.7% (n = 925), 82.2% (n = 82), 77.4% (n = 373), and 60.3% (n = 221), respectively (Fig. 3A). Because the difference of recurrence-free proportion curves between the VPI+/MVI− and VPI−/MVI+ groups was not statistically significant ($P = 0.177$), we divided the cases into 3 groups according to the number of the risk factors for VPI and MVI (Fig. 3B). This showed a significant separation in the recurrence-free proportion curves between these 3 groups: both VPI and MVI absent (0), either VPI or MVI present (1), and both VPI and MVI present (2). These findings were also observed among the overall survival curves (Supplemental Figs 1A and B, available at http://links.lww.com/SLA/A545).

We then analyzed the recurrence-free proportion of patients stratified by T subgroup according to tumor size (T1a, T1b, T2a, T2b, and T3; ≤2 cm, ≤3 cm, ≤5 cm, ≤7 cm, and >7 cm, respectively) and VPI and MVI status (both absent, either present, or both present; 0/1/2). All 13 patients with T4 disease had MVI and 12 had VPI. The proportion of patients with a 5-year recurrence-free period with T1a_0, T1b_0, T2a_0, T2b_0, T1a_1, T1b_1, T2a_1, T3_0, T1a_2, T1b_2, T3_1, T2b_1, T2b_2, T2a_2, T3_2, and T4 were 92.2%, 89.6%, 87.8%, 75.9%, 72.2%, 64.8%, 61.9%, 58.2%, 50.9%, 50.6%, 49.4%, 47.5%, 44.8%, 43.6%, 38.8%, and 32.4%, respectively (Supplemental Figs. 2A, B, C, D, and E, available at http://links.lww.com/SLA/A545). The groups with small tumor size and no VPI and MVI (T1a_0, T1b_0, and T2a_0) showed the best recurrence-free proportions. In contrast, the T1a_1, T1b_1, and

TABLE 3. Prognostic Significance of Recurrence in All Patients with T1-4N0-2M0 NSCLC (Univariate and Multivariate Cox Regression Analyses)

		Univariate Analysis			Multivariate Analysis		
Variable	**Risk Factors**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age, yrs	>65	1.22	1.07–1.39	0.004	1.27	1.11–1.45	<0.001
Sex	Male	1.41	1.22–1.63	<0.001	0.93	0.76–1.14	0.476
Smoking history	Ever smokers	1.51	1.30–1.76	<0.001	1.18	0.94–1.47	0.149
CEA, ng/mL	>5	1.84	1.61–2.10	<0.001	1.23	1.07–1.42	0.003
Histology	Nonadenocarcinoma	1.22	1.06–1.40	<0.001	1.40	1.20–1.63	<0.001
Tumor size, cm	>3	2.37	2.07–2.71	<0.001	1.26	1.08–1.46	0.003
pN status	pN1–2	4.40	3.85–5.04	<0.001	2.58	2.23–3.00	<0.001
VPI	Present	2.91	2.54–3.33	<0.001	1.51	1.30–1.75	<0.001
MVI	Present	4.88	4.11–5.82	<0.001	2.78	2.29–3.40	<0.001

CI indicates confidence interval.

TABLE 4. Clinicopathological Characteristics of Pathological Stage I Patients

Variable	**n (%)**
Age, yrs	
Median 66 (range: 22–89)	
≤65	780 (48.7)
>65	821 (51.3)
Sex	
Female	684 (42.7)
Male	917 (57.3)
Smoking history	
Never smokers	620 (38.7)
Ever smokers	969 (60.5)
NA	12 (0.7)
CEA, ng/mL	
≤5	1137 (71.0)
>5	455 (28.4)
NA	9 (0.6)
Surgery	
Segmentectomy	24 (1.5)
Lobectomy	1574 (98.3)
Pneumonectomy	3 (0.2)
Histology	
Adenocarcinoma	1192 (74.5)
Squamous cell carcinoma	282 (17.6)
Large cell carcinoma	65 (4.1)
Adenosquamous carcinoma	26 (1.6)
Others	36 (2.2)
Tumor size, cm	
≤3	1177 (73.5)
>3	424 (26.5)
VPI	
Absent	1298 (81.1)
Present	303 (18.9)
MVI	
Absent	1007 (62.9)
Present	594 (37.1)

T2a_1 subgroups had poorer outcomes, which were comparable with those of the T2b_0 subgroup. Poor outcomes equivalent to those seen in the T3_0/1 groups were observed in groups with a small tumor size and both VPI and MVI. The T3_2 group showed the poorest outcome after the T4 group.

DISCUSSION

In patients with T1-4N0-2M0 NSCLC, the overall survival rate and recurrence-free proportion for patients with MVI were significantly lower than those for patients without MVI. Multivariate analysis showed that MVI was the most powerful predictor of recurrence, with its HR of 2.78 being greater than that of pN (HR 2.58). When only patients with pathological stage I disease were analyzed using multivariate analysis, MVI and VPI were the 2 most powerful factors for recurrence (MVI, HR: 2.74; VPI, HR: 1.84).

VPI is incorporated in the current seventh edition of TNM classification. In this study, we demonstrated that MVI was also a strong pathological prognostic factor. The T1-2 groups with both MVI and VPI showed poor outcomes equivalent to those of the T3 groups with none or either of these factors. Therefore, we propose that both MVI and VPI should be incorporated in the new edition of TNM staging system.

Some studies have demonstrated the prognostic impact of BVI and LVI, and MVI has been reported to be a poor prognostic factor in NSCLC patients.[6–9,11–14] We used elastin, lymphatic endothelial, and H&E stains to histologically differentiate between BVI and LVI. While these stains are helpful adjuncts, differentiation can still be difficult in capillary areas. Therefore, several investigators have examined BVI and LVI collectively as MVI,[6,8,9] and we decided to use MVI as an objective reproducible morphological criterion.

As indicated in Supplemental Figures 2A–E (available at http://links.lww.com/SLA/A545), the T1a_0, T1b_0, and T2a_0 subgroups showed the best prognosis in the current study. The T1a_1, T1b_1, and T2a_1 subgroups showed poorer prognosis, consistent with the T2b_0 subgroup, whereas the T1a-2b_2 and T2b_1 subgroups had poor prognosis, equivalent to the T3_0/1 subgroups. The T3_2 subgroup had the poorest prognosis after the T4 subgroup. Therefore, we propose that if either MVI or VPI is present, the tumor size subgroups of T1 should be upstaged to T2; T1 with both MVI and VPI should be upstaged to T3; T2 with either or both MVI and VPI should be upstaged to T3 (except T2a with either MVI or VPI); and T3 with both MVI and VPI should be upstaged to T4. These suggestions are summarized in Tables 6 and 7.We show the recurrence-free proportion curves by the current classification and our proposal for T factor (Figs. 4A and B). The current T1–3 groups include the cases of poor prognosis with MVI and/or VPI, who might require additional therapy.

Platinum-based adjuvant chemotherapy has been shown in randomized controlled trials to improve the survival of patients with stage II and IIIA NSCLC who underwent surgery.[15,16] In Japan, oral uracil-tegafur adjuvant chemotherapy for patients with stage IB adenocarcinoma is recommended as the standard treatment.[17,18] The results of the current study indicate that NSCLC patients with small sized tumors with both or either MVI and VPI risk factors would be good candidates for adjuvant therapy.

TABLE 5. Prognostic Significance of Recurrence in pathological Stage I Patients (Univariate and Multivariate Cox Regression Analyses)

		Univariate Analysis			Multivariate Analysis		
Variable	**Risk Factors**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age, yrs	>65	1.49	1.19–1.89	<0.001	1.37	1.08–1.75	0.009
Sex	Male	1.65	1.30–2.10	<0.001	1.01	0.72–1.40	0.940
Smoking history	Ever smokers	1.97	1.53–2.56	<0.001	1.46	1.01–2.10	0.042
CEA, ng/mL	>5	1.74	1.37–2.19	<0.001	1.22	0.96–1.56	0.101
Histology	Nonadenocarcinoma	1.58	1.24–2.01	<0.001	0.94	0.72–1.23	0.944
Tumor size, cm	>3	1.99	1.58–2.52	<0.001	1.17	0.91–1.50	0.231
VPI	Present	3.00	2.37–3.80	<0.001	1.84	1.43–2.37	<0.001
MVI	Present	3.73	2.94–4.76	<0.001	2.74	2.10–3.60	<0.001

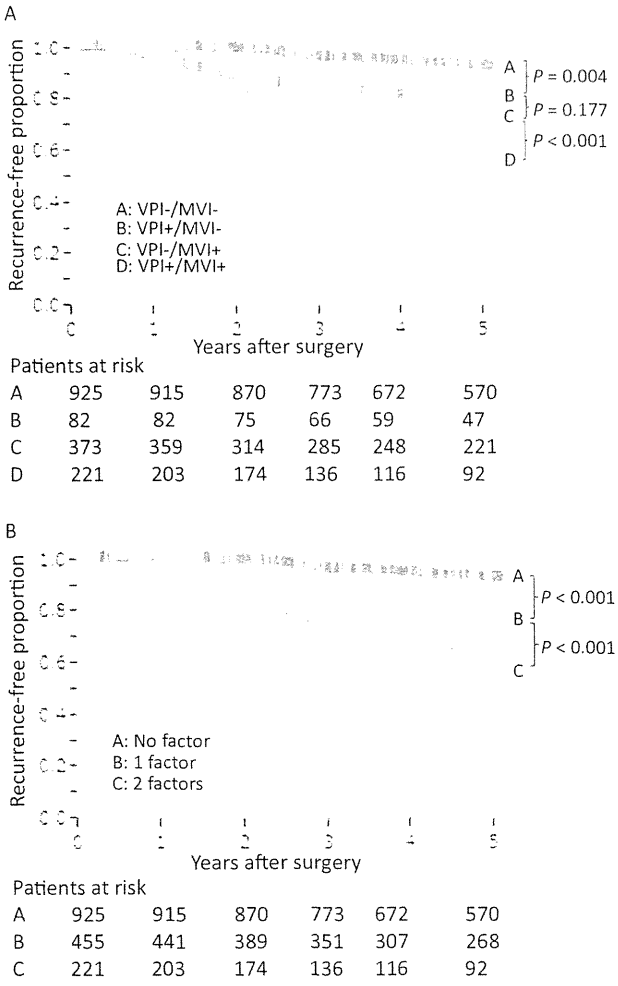

FIGURE 3. A, Recurrence-free proportion curves of patients with or without MVI and VPI; B, recurrence-free proportion curves of patients according to the number of the risk factors.

TABLE 6. Incorporation of VPI and MVI Into T Classification

Proposed T	**Tumor Size, cm**	**No. Risk Factors (VPI/MVI)**	**No. Patients (%)**	**Recurrence-Free Proportion at 5 Yrs, %**
T1	≤5	0	1001 (37.7)	90.5
T2	≤5	1	741 (27.9)	65.8
	>5, ≤7	0		
T3	≤7	2	702 (26.4)	47.1
	>5, ≤7	1		
	>7	0		
	>7	1		
T4	>7	2	213 (8.0)	38.6

TABLE 7. Incorporation of VPI and MVI Into T Classification

Current (7th) T Classification	**Tumor Size, cm**	**No. VPI and MVI Risk Factors**	**No. Patients (%)**	**Our Proposed T**
T1a	≤2	0	474 (17.8)	T1
	≤2	1	173 (6.5)	T2
	≤2	2	51 (1.9)	T3
T1b	>2, ≤3	0	337 (12.7)	T1
	>2, ≤3	1	258 (9.7)	T2
	>2, ≤3	2	126 (4.7)	T3
T2a	>3, ≤5	0	190 (7.2)	T1
	>3, ≤5	1	290 (10.7)	T2
	>3, ≤5	2	265 (10.0)	T3
T2b	>5, ≤7	0	20 (0.8)	T2
	>5, ≤7	1	68 (2.6)	T3
	>5, ≤7	2	95 (3.6)	T3
T3	>7	0	26 (1.0)	T3
	>7	1	71 (2.7)	T3
	>7	2	200 (7.5)	T4

Because our study was a retrospective study of a generally homogenous ethnic group performed in a single institution, we need to collect more clinicopathological data from a diverse population and validate our proposal on the basis of these data. However, this study included a large number of patients and showed that MVI and VPI were both strong prognostic factors. Therefore, we consider that MVI should be evaluated in the T classification for the possible incorporation in the next revision of the TNM staging system.

CONCLUSIONS

This study demonstrated that MVI was a strong and independent recurrence risk factor in patients with pathological T1-4N0-2M0 NSCLC. Further data on the prognostic impact of MVI should be collected for the next revision of the TNM staging system.

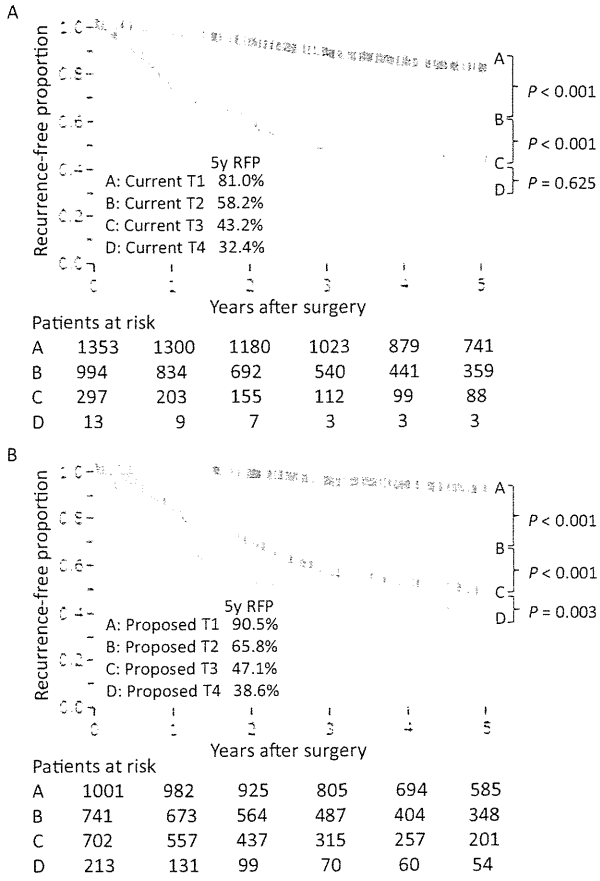

FIGURE 4. A, Recurrence-free proportion curves according to the current T classification. B, Recurrence-free proportion curves according to our proposal for T classification.

REFERENCES

1. Shimizu K, Yoshida J, Nagai K, et al. Visceral pleural invasion classification in non-small cell lung cancer: a proposal on the basis of outcome assessment. *J Thorac Cardiovasc Surg.* 2004;127:1574–1578.
2. Travis WD, Brambilla E, Rami-Porta R, 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;3:1384–1390.
3. Kawase A, Yoshida J, Miyaoka E, et al. Visceral pleural invasion classification in non-small-cell lung cancer in the 7th edition of the tumor, node, metastasis classification for lung cancer: validation analysis based on a large-scale nationwide database. *J Thorac Oncol.* 2013;8:606–611.
4. Yoshida J, Nagai K, Asamura H, et al. Visceral pleura invasion impact on non-small cell lung cancer patient survival: its implications for the forthcoming TNM staging based on a large-scale nation-wide database. *J Thorac Oncol.* 2009;4:959–963.
5. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. *J Thorac Oncol.* 2007;2:706–714.
6. Tsuchiya T, Hashizume S, Akamine S, et al. Upstaging by vessel invasion improves the pathology staging system of non-small cell lung cancer. *Chest.* 2007;132:170–177.
7. Brechot JM, Chevret S, Charpentier MC, 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;78:2111–2118.
8. Ruffini E, Asioli S, Filosso PL, 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;6:319–326.
9. Miyoshi K, Moriyama S, Kunitomo T, et al. Prognostic impact of intratumoral vessel invasion in completely resected pathologic stage I non-small cell lung cancer. *J Thorac Cardiovasc Surg.* 2009;137:429–434.
10. Travis WD, Brambilla E, Müller-Hermelink HK, et al. *Tumours of the Lung, Pleura, Thymus and Heart.* Lyon, France: IARC Press; 2004.
11. Hishida T, Yoshida J, Maeda R, et al. Prognostic impact of intratumoural microvascular invasion and microlymphatic permeation on node-negative non-small-cell lung cancer: which indicator is the stronger prognostic factor? *Eur J Cardiothorac Surg.* 2013;43:772–777.
12. Maeda R, Yoshida J, Ishii G, et al. Prognostic impact of intratumoral vascular invasion in non-small cell lung cancer patients. *Thorax.* 2010;65:1092–1098.
13. Suzuki K, Nagai K, Yoshida J, 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;86:1976–1984.
14. 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.* 2011;6:48–54.
15. Winton T, Livingston R, Johnson D, et al. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. *N Engl J Med.* 2005;352:2589–2597.
16. Arriagada R, Bergman B, Dunant A, et al. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *N Engl J Med.* 2004;350:351–360.
17. Kato H, Ichinose Y, Ohta M, et al. A randomized trial of adjuvant chemotherapy with uracil-tegafur for adenocarcinoma of the lung. *N Engl J Med.* 2004;350:1713–1721.
18. Hamada C, Tanaka F, Ohta M, et al. Meta-analysis of postoperative adjuvant chemotherapy with tegafur-uracil in non-small-cell lung cancer. *J Clin Oncol.* 2005;23:4999–5006.