characteristics of cancer cells coexisting with PDPN-CAFs and found an anatomical correlation between PDPN-CAFs and Ezrin-expressing cancer cells. This is the first report to investigate the phenotypes of cancer cells in a microenvironment composed of a specific subpopulation of CAFs, i.e., PDPN-CAFs, and to suggest the implications of microenvironmental heterogeneity within lung adenocarcinoma.

Table 3 Staining scores of cancer cells in PDPN-CAFs negative area and positive area

Antibody	Staining score of cancer cells around CAFs[a]		*p* value
	PDPN-CAFs negative area	PDPN-CAFs positive area	
Ezrin	42.5 (0–100)	70 (30–100)	<0.01
E-Cadherin	72.5 (30–180)	80 (20–185)	0.76

PDPN-CAFs podoplanin-expressing cancer-associated fibroblast

[a] Median (range)

Ezrin is a member of the ERM (Ezrin–Radixin–Moesin) protein family, which provides a physical link between F-actin and cell membrane-associated proteins (Bretscher et al. 2002; Louvet-Vallee 2000; McClatchey 2003). ERM proteins are associated with several adhesion molecules, such as CD44, and their functions are essential for fundamental cellular processes, including cell adhesion and motility (Pujuguet et al. 2003; Tsukita et al. 1994; Xu and Yu 2003). Reportedly, Ezrin is strongly expressed in many types of tumors (Cui et al. 2010; Di Cristofano et al. 2010; Ma et al. 2013; Tynninen et al. 2004; Zhang et al. 2012). In

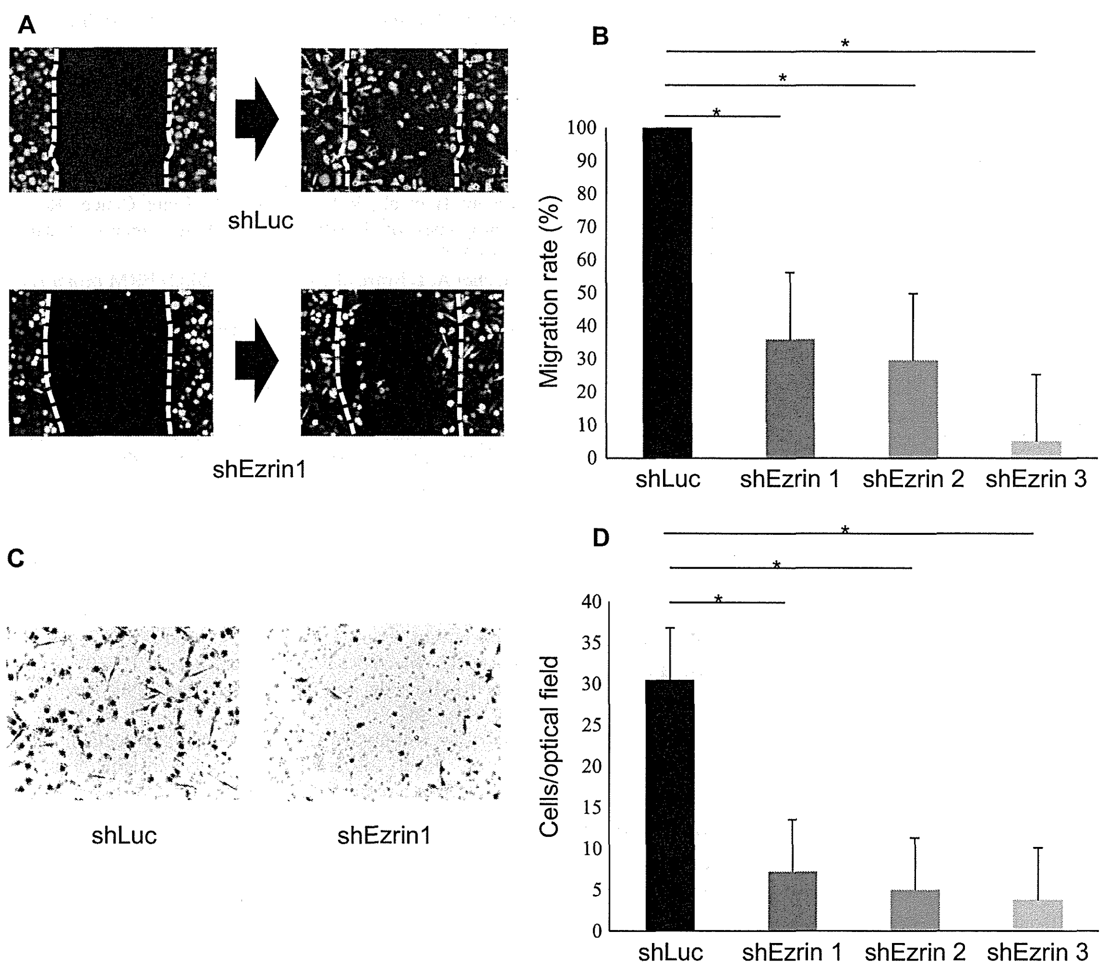

Fig. 4 Wound healing assay and Matrigel invasion assay using Ezrin-knockdown PC-9 cells. **a** Wound healing assay. The green cells are shLuciferase or shEzrin 1-induced PC-9 cells at 7 h after well scratching. The white dotted lines represent the scratch lines. **b** Migration rate of the cells into the central area of the wound as an indicator of migration activity using shEzrin 1 to 3-induced PC-9 cells or shLuciferase-induced PC-9 cells. **c** Matrigel invasion assay. shLuciferase-induced or shEzrin 1-induced PC-9 cells that had passed through a filter are shown. **d** Number of cells that had invaded through the filter into the lower chamber as an indicator of invasive activity using shEzrin 1 to 3-induced PC-9 cells or shLuciferase-induced PC-9 cells. *$p < 0.05$

lung cancer, Ezrin expression in cancer correlated significantly with lymphatic metastasis and advanced TNM stage (Li et al. 2012). Similarly, in our study, Ezrin expression in cancer cells ("positive" was judged as over 50 % of cancer cells) showed higher tendency of pleural invasion (positive vs. negative: $p = 0.05$) (Supplementary Table 4).

In lung adenocarcinoma, the high level of Ezrin expression is reportedly associated with wide therapeutic applications in the treatment of human lung cancer in studies using the human lung carcinoma cell line 95D, with connections to the promotion of morphological change, migration, growth, and invasiveness (Chen et al. 2013). These findings are consistent with our present in vitro results. Therefore, Ezrin is thought to be a key regulatory molecule in invasiveness. These findings may partly explain why PDPN-CAF (+) adenocarcinoma cases had higher degrees of vascular invasion, node metastasis, and pleural invasion.

Activated Ezrin in cancer cells can link various plasma membrane proteins to the actin cytoskeleton (Fehon et al. 2010). Activated Ezrin also binds and sequesters RhoGDI (Rho GDP-dissociation inhibitor) (Hirao et al. 1996; Maeda et al. 1999; Takahashi et al. 1997), thereby initiating the activation of RhoA and maintaining Ezrin activation. On the other hand, we previously reported that PDPN-expressing fibroblasts exhibited higher levels of RhoA activity (Ito et al. 2012b). Taking these into consideration, RhoA activity might increase in both PDPN-CAFs and Ezrin-expressing cancer cells within the same area; therefore, Rho-inhibitor might appear to have great potential as a new therapeutic target for cancer microenvironment composed of PDPN-CAFs.

Meanwhile, the molecular mechanisms responsible for inducing the expression of PDPN in CAFs and the expression of Ezrin in cancer cells have been unclear. As a PDPN expression-promoting factor, TGF-β1 has been reported in an oral squamous cell carcinoma cell line (Ohta et al. 2013) and a human fibrosarcoma cell line, HT1080 (Suzuki et al. 2008). TGF-β1 has also been reported to be an Ezrin expression-promoting factor in human trophoblast derived from placentae and the human choriocarcinoma cell line JEG-3 (Karmakar and Das 2004). A tumor microenvironment composed of PDPN-CAFs and Ezrin-expressing cancer cells might exist under TGF-β1-rich conditions. In lung cancer, Hasegawa et al. (2001) reported a significant correlation between TGFβ1 expression level and poor prognosis in patients with adenocarcinoma, which might also support hypothesis mentioned above. Alternatively, PDPN-CAFs might induce Ezrin expression in cancer cells or vice versa; this issue will require further examination in the future.

In conclusion, our study demonstrated that Ezrin expression is a characteristic phenotype of cancer cells in microenvironments containing PDPN-CAFs in lung adenocarcinoma. The current results also indicate the presence of microenvironmental heterogeneity within solid tumors and suggest the biological significance of the unique microenvironment created by Ezrin-overexpressing cancer cells and PDPN-overexpressing CAFs.

At present, not only cancer cells, but also CAFs and their interactions are attracting attention as potential therapeutic targets (Hofheinz et al. 2003; Kraman et al. 2010; Scott et al. 2003). Our discoveries in the present study might serve as a foundation for the development of promising cancer therapies targeting for both cancer cells and CAFs.

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

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Key words

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

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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

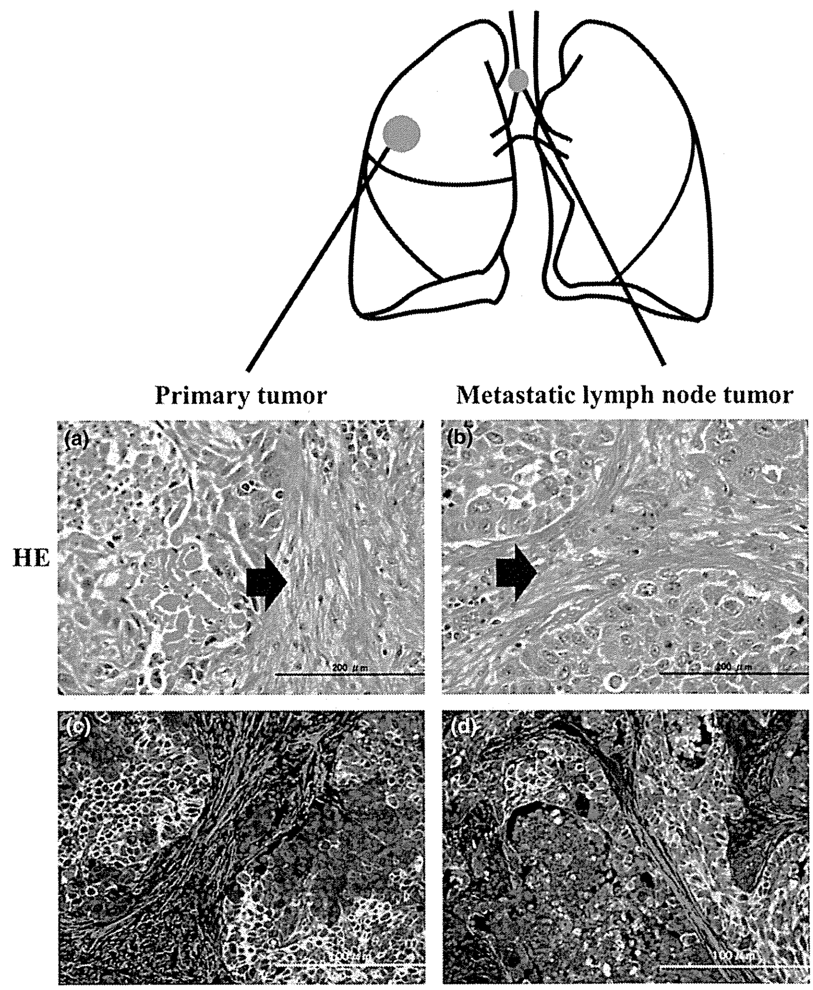

Fig. 1. Staining with H&E and double immunofluorescence staining of tumor cells in a primary and a metastatic lymph node tumor in a patient with squamous cell carcinoma of the lung. (a, b) H&E staining in the primary tumor (a) and the metastatic lymph node tumor (b). Arrows indicate cancer-associated 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-

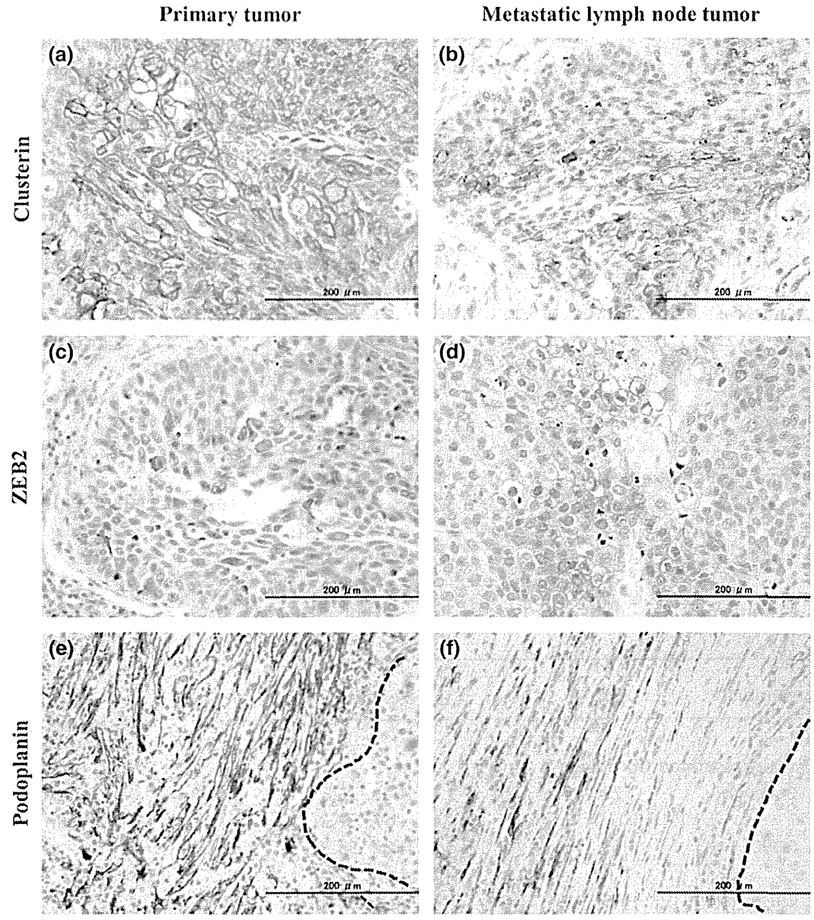

Fig. 2. Immunohistochemical staining of tumor cells in a primary tumor and a metastatic lymph node tumor in a patient with squamous cell carcinoma of the lung. (a,b) Clusterin expression of cancer cells in the primary tumor (a) and the metastatic lymph node tumor (b). (c,d) ZEB2 expression of cancer 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.

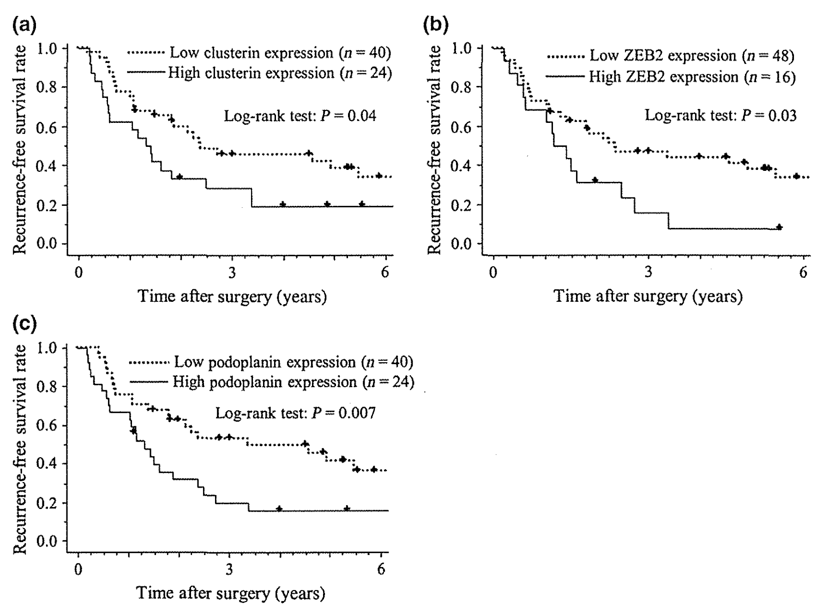

Fig. 3. Kaplan–Meier recurrence-free survival (RFS) curve for patients with resected pathological N2 squamous cell carcinoma of the lung according to immunohistochemical staining. (a) Kaplan–Meier RFS curve according to clusterin expression of cancer cells in metastatic lymph node tumors. (b) Kaplan–Meier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

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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

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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.

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Survival of 1737 lobectomy-tolerable patients who underwent limited resection for cStage IA non-small-cell lung cancer†

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Abstract

OBJECTIVES: A precise preoperative diagnosis of 'very early' lung carcinoma may identify patients who can undergo curative surgery with limited resections.

METHODS: Data from a multi-institutional project were collected on 1737 patients who had undergone limited resections (segmentectomy or wedge resection) for T1N0M0 non-small-cell carcinomas. As it was expected, this study was predominantly including ground glass nodules. Computed tomography was used to obtain the ratio of consolidation to the maximal tumour diameter to determine invasive potential of the tumours. Overall and disease-free survivals and recurrence-free proportions were analysed.

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RESULTS: Median age was 64 years. Mean maximal diameter of the tumours was 1.4 ± 0.5 cm. Overall and recurrence-free survivals after limited lung resection were 94.0 and 91.1% at 5 years, respectively. Recurrence-free proportions were 93.7% at 5 years. Unfavourable prognostic factors in overall survival were lymph node metastasis, interstitial pneumonia, male gender, older age, comorbidities (cardiac disease, diabetes etc.) and consolidation/tumour ratio (C/T) ≤ 0.25. C/T ≤ 0.25 predicted good outcomes especially in cT1aN0M0 disease. In a subclass analysis of cT1N0M0 squamous cell carcinomas, wedge resection was the only unfavourable prognostic factor in both overall and disease-free survivals.

CONCLUSIONS: If the patient was 75 years old or younger and was judged fit for lobectomy, limited resection for cStage I non-small-cell lung cancer (NSCLC) showed excellent outcomes and was not inferior to the reported results of lobectomy for small-sized NSCLC. The carcinomas with C/T ≤ 0.25 rarely recur and are especially good candidates for limited resection.

Keywords: Lung cancer • Limited resection • Segmentectomy • Wedge resection

INTRODUCTION

In early-stage non-small-cell lung cancer (NSCLC), a curative surgical resection has been considered to be the determinant of prognosis [1, 2]. Lobectomy is the standard surgical procedure for early-stage NSCLC [3]. Limited resection has been associated with a higher risk of local recurrence in a randomized controlled study reported in 1995 [3]. However, excellent survival data for limited resections have been reported in patients with small-sized NSCLC [4–7]. Moreover, a recent evaluation of the surveillance, epidemiology and end results (SEER) registry with a large number of patients suggested that limited resection may be an adequate alternative for stage IA NSCLC patients with tumours ≤1 cm in size [8].

Recent widespread use of computed tomography (CT) for lung cancer screening has led to increased detection of 'very early' NSCLC [9]. To predict pathological non-invasiveness by radiological findings, the Japan Clinical Oncology Group (JCOG) conducted a prospective radiological and pathological study of clinical stage IA lung cancers (JCOG0201) [10, 11]. As a result, a consolidation/tumour ratio (C/T) of 0.25 or less in cT1a (≤2.0 cm) was reported to be a good predictor of non-invasive adenocarcinoma [10, 11] in studies of predominantly ground glass nodules (GGNs). Using this criterion, a phase II study was conducted of limited surgical resections for peripheral early lung cancers diagnosed by thin-section CT findings (JCOG0804/WJOG4507L). Although patient enrolment ended in 2011, more time will be needed before the results of the study are available.

In practice, limited resection has been widely used for pure or part-solid GGNs especially for small lesions [12, 13]. The aim of this retrospective study was to compile clinical data of a large number of patients who underwent limited resections for clinical stage I NSCLC. To more accurately determine the efficacy of limited resection, our study was limited to patients 75 years old or younger and who were diagnosed fit for lobectomy.

MATERIALS AND METHODS

The Japanese Association for Chest Surgery collected clinical data in a retrospective multi-institutional analysis of limited resections (segmentectomy or wedge resection) for NSCLC patients who were expected to tolerate lobectomy and were 75 years old or younger at the time of operation. Registration enrolment criteria for the patients were: 30 or more consecutive patients with clinical stage I NSCLC undergoing limited resections (segmentectomy or wedge resection) at each institution, 60 or more months of median follow-up period, age ≤75 years at operation and physically fit for lobectomy as diagnosed by the physician. The newest the Union Internationale Contre le Cancer the tumour, node, and metastases staging classification of version 7 was used in this study.

To grasp the present conditions and genuine prognosis of limited resection in Japan, all clinical stage I disease and all pathological types except small-cell carcinomas were included. In this study, lymph node dissection was not queried. In wedge resection, evaluation of lymph node metastasis was not possible. The cases with incomplete pathological evaluation of lymph nodes were expressed by pNx. Also the criteria for patients to fit lobectomy were not set because they varied between institutions. We aimed to clarify the prognosis of the patients who were thought to be curable by intentional limited resection. As we thought the detail criteria for patients to fit lobectomy were not necessary for the analysis of intentional curable limited resection but palliative limited resection, we did not analyse them in this study.

Twenty-six institutions participated in the present study, and 2212 patients were enrolled. They underwent limited resections between August 1993 and July 2012. Two hundred and forty-nine patients were excluded from analysis for the following reasons: small-cell carcinoma, no data of pulmonary function test, low pulmonary function [e.g. forced expiratory volume in one second (FEV1.0) <40% of predicted level], dissemination found at operation or positive cancer cells at the cut end of limited resection. At first, synchronous and metachronous multiple lung cancers were included in the analysis but then these lesions were excluded. The remaining 1737 patients were analysed.

This study was approved by the Institutional Review Boards of the Nagoya City University Hospital and participating institutions. Patient demographics and lung cancer-related factors including the number of comorbidities (diabetes mellitus, cardiac disease etc.), especially comorbidities of interstitial pneumonia (IP) including idiopathic pulmonary fibrosis, lung function [%VC (vital capacity) and FEV1%], radiological findings on CT, location of the nodule (outer 1/3 or inner 2/3), pathology, clinical and pathological TNM status, tumour size, number of nodules and operation performed (segmentectomy/wedge resection), were registered.

As it was expected, this study predominantly included ground glass nodules. A tumour was radiologically classified into two groups; as an invasive group if the ratio of the consolidation (C) to the maximal tumour diameter (T) (C/T ratio) was more than 0.25 (C/T > 0.25), and a non-invasive group (C/T ratio was ≤ 0.25: C/T ≤ 0.25) [10, 11]. This criterion was in accordance with the definition of radiological noninvasive lung cancer reported by the JCOG in the JCOG0201 study reports [10, 11].

Statistical analysis

The overall survival length was defined as the interval in months from the date of surgery to the date of death from any cause and patients who were alive were censored at the date of the last follow-up. Recurrence-free survival was defined as the interval from the date of surgery to the date of recurrence or death from any cause and alive patients without recurrence were censored at the date of the last follow-up. Recurrence-free proportion was defined as the proportion of patients free from recurrence regardless of survival or death and alive patients without recurrence were censored at the date of the last follow-up. In addition, the patients who died of any cause other than lung cancer were censored at the date of the last follow-up.

The probability of survival or proportion was estimated by the Kaplan-Meier method. The statistical significance of differences in survival between categorized groups was evaluated by the log-rank test. The significance of differences between categorized groups was evaluated using Pearson's χ^2 test. Cox regression analysis was used to identify prognostic factors. Statistical significance was defined as $P < 0.05$. The EZR software on a personal computer was used for the analysis [14]. The data were analysed at the Nagoya City University Data Center.

RESULTS

The characteristics of 1737 patients are given in Table 1 and Fig. 1. In brief, the median age was 63 years, and women were slightly predominant. The main population of patients in this study were without comorbidities especially without IP, with small maximal diameters of tumours (≤2.0 cm), and diagnosed as cT1aN0M0. Tumours were located in the outer one-third of the lung parenchyma. Almost half of the tumours were diagnosed radiologically as invasive (C/T > 0.25). Segmentectomy was performed in almost two-third cases. Most of the lung cancers were diagnosed as adenocarcinoma with pT1a N0 or pNx.

Prognosis

The mean observation period was 71.7 months (median: 65.8 months). During the observation period 141 patients died: 70 patients from lung cancer and 71 patients from other causes. Recurrence occurred in 122 patients. The recurrences were distant metastases in 46 patients (37.7%), local recurrence in 30 (24.6%), lymph node recurrence in 28 (23.0%) and pleural dissemination in 18 (14.8%). This local recurrence meant a recurrence lesion, which

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Table 1: Patient characteristics

Factors	Characteristics	n (%)
Age	Median 64 (20–75)	
	<65	893 (51.4)
	65≤	844 (48.6)
Gender	Women	950 (54.7)
	Men	787 (45.3)
Number of comorbidity	0	1208 (69.5)
	1	427 (24.6)
	2 or more	102 (5.9)
Comorbidity of interstitial pneumonia	–	1706 (98.2)
	+	31 (1.8)
%VC	≥80%	1653 (95.2)
	<80%	84 (4.8)
FEV1%	≥70%	1400 (80.6)
	<70%	337 (19.4)
Maximal diameter (MD)	Mean 1.4 ± 0.5 cm	
	MD ≤ 1.0 cm	551 (31.7)
	1.0 cm < MD ≤ 2.0 cm	1026 (59.1)
	2.0 cm < MD	160 (9.2)
Location	Outer	1250 (72.0)
	Inner	234 (13.5)
	unknown	253 (14.6)
Radiological findings (C/T)	C/T > 0.25	915 (52.7)
	C/T ≤ 0.25	810 (46.6)
	unknown	14 (0.8)
cStage	cT1aN0M0	1576 (90.7)
	cT1bNoMo	161 (9.3)
Performed operation	Segmentectomy	1094 (63.0)
	Wedge resection	643 (37.0)
Pathology	Adenocarcinoma	1586 (91.3)
	SqCC	100 (5.8)
	Others	51 (2.9)
pT factors	pT1a	1497 (86.2)
	pT1b	131 (7.5)
	pT2-3	109 (6.3)
pN factors	pN0 or Nx	1715 (98.7)
	pN1 or 2	22 (1.3)

%VC: vital capacity; FEV1%: forced expiratory volume in one second; C/T: the ratio of the consolidation (C) to the maximal tumour diameter (T); HR: hazard ratio; CI: confidence interval.

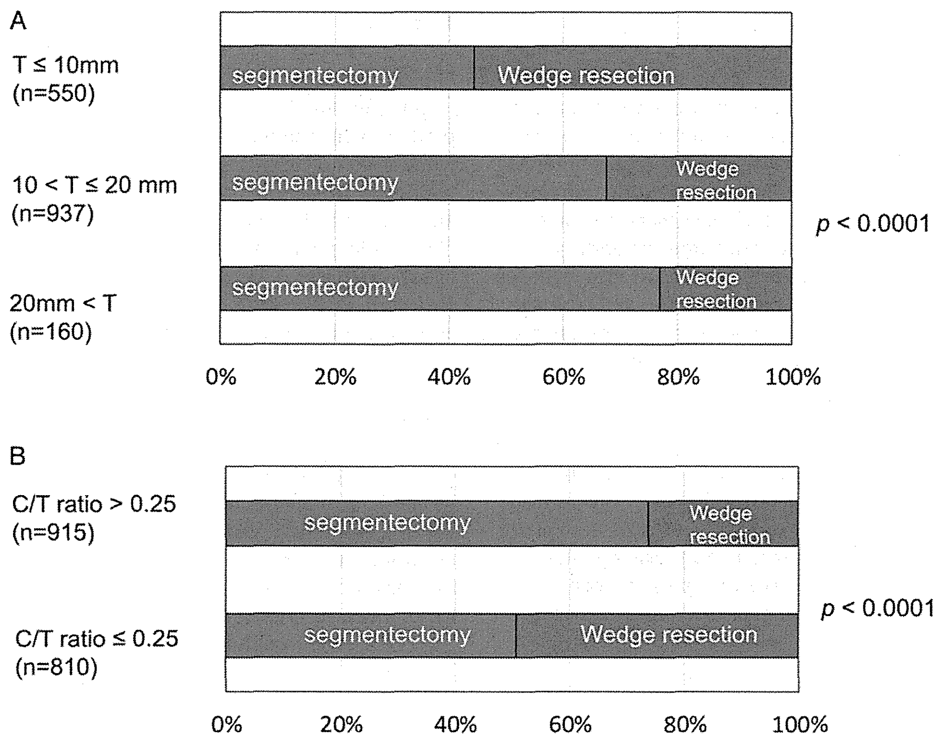

Figure 1: Preoperative tumour factors and differences in surgical procedures, segmentectomy and wedge resection. **(A)** Maximal diameter of the tumours (T) and differences in surgical procedures. **(B)** Radiological findings of C/T ratio and differences in surgical procedures. C/T ratio: ratio of the maximal diameters of tumour (T) and consolidation (C).

could be distinguished from the lymph node recurrence and located near the stamp of the bronchus or the lung parenchyma. The overall survival of the 1737 patients who underwent limited resections was 94.0% at 5 years (95% confidence interval (CI): 92.6–95.1%; Fig. 2A). The disease-free survival was 91.1% at 5 years (95 CI: 89.5–92.4%) (Fig. 2B). The recurrence-free proportion was 93.7% at 5 years (95 CI: 92.4–94.8%) (Fig. 2C). The yearly recurrence rate was calculated as the number of patients with recurrence divided by the number of patients at risk at the beginning of each year (Fig. 3). Most recurrences were diagnosed in the first 3 years of follow-up after the surgery (yearly recurrence rate 1.50–1.64%). From 4 to 7 years, the recurrence proportion was <1%. Then from 8 to 9 years, a small peak of the recurrence may be present. The overall survival in the 122 patients with recurrences was 57.4% at 5 years after surgery (95% CI: 47.7–65.9%).

Cox's multivariate analysis was performed for prognostic factors for overall survival, disease-free survival, recurrence-free proportion and proportion free from death by other causes (Table 2). In summary, unfavourable prognostic factors in overall survival were pathologically confirmed lymph node metastasis, male gender, comorbidity of IP, older age (≥65), advanced pathological T factor (pT2a-3), C/T > 0.25, and presence of any comorbidities. Unfavourable prognostic factors in disease-free survival were pathologically confirmed lymph node metastasis, male gender, C/T > 0.25, advanced pathological *T* factor (pT2a-3), low %VC, comorbidity of IP, older age (≥65) and presence of any comorbidities. Unfavourable prognostic factors in recurrence-free proportion were pathologically confirmed lymph node metastasis, C/T > 0.25, comorbidity of IP, advanced pathological T factors (pT2a-3), male gender, low %VC and presence of any comorbidities. Unfavourable prognostic factors in the proportion free of death from other causes were older age (≥65), male gender and squamous cell carcinoma pathology (SqCC).

Subclass analysis

To compare the results of the present study to a recent study reporting the results of lobectomy for small-sized NSCLC, subclass analysis was performed in 1538 patients with a cT1aN0M0 disease of a single lesion without IP. The overall survival in 1538 patients was 94.7% at 5 years (95 CI: 93.3–95.8%) (Fig. 4A). The disease-free survival was 92.2% at 5 years (95 CI: 90.7–93.5%) (Fig. 4B). The recurrence-free proportion was 95.0% at 5 years (95 CI: 93.7–96.0%) (Fig. 4C).

Among factors available preoperatively, the CT finding according to the C/T ratio was the prognostic factor that had the greatest effect on recurrence (Table 2). The overall survival of patients of C/T > 0.25 ($n = 755$) and C/T ≤ 0.25 ($n = 783$) tumours was 92.7 (95 CI: 90.7–94.7%) and 96.7 (95 CI: 95.4–98.2%) at 5 years, respectively ($P < 0.0001$; Fig. 5A). The disease-free survival of patients of C/T > 0.25 and C/T ≤ 0.25 was 88.2 (95 CI: 85.5–90.4%) and 96.5% at 5 years (95 CI: 94.7–97.7%), respectively ($P < 0.0001$; Fig. 5B). The recurrence-free proportion in patients of C/T > 0.25 and C/T ≤ 0.25 was 91.6 (95 CI: 89.3–93.4%) and 98.5% at 5 years (95 CI: 97.3–99.2%), respectively ($P < 0.0001$; Fig. 5C).

SqCC, the second major pathological type, was analysed separately. One hundred patients with cT1N0M0 SqCC diseases were assessed. The mean tumour size was 1.6 ± 0.6 cm. The overall and disease-free survivals were 83.5 and 76.3% at 5 years, respectively

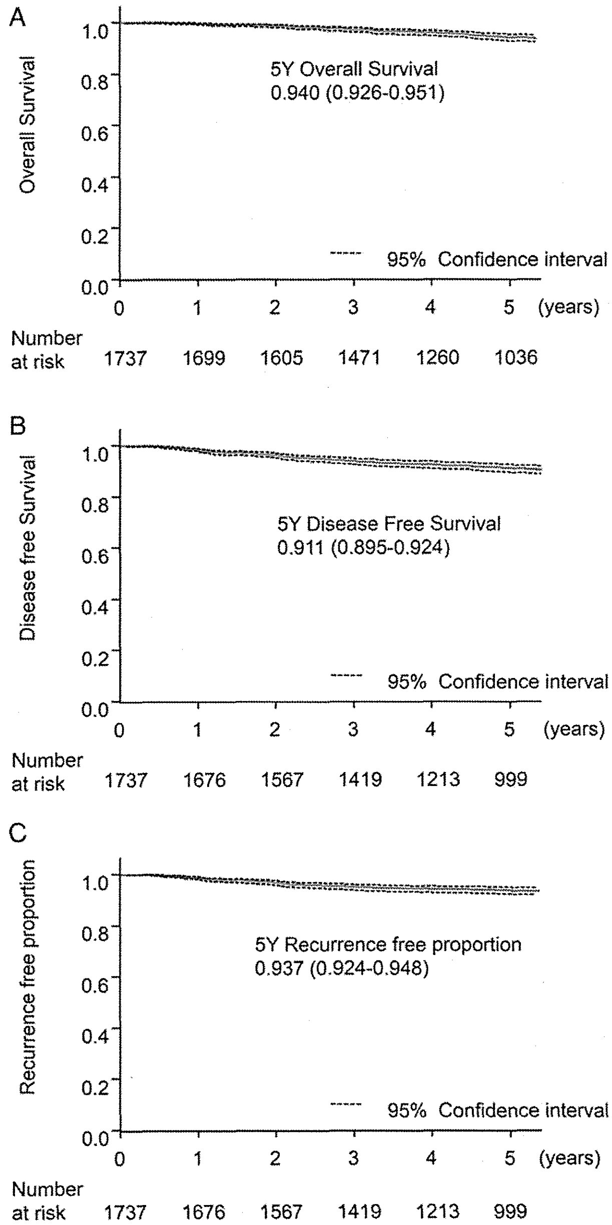

Figure 2: Survival and recurrence data of the entire 1737 patients. (**A**) Five-year overall survival. (**B**) Five-year disease-free survival. (**C**) Five-year recurrence-free proportion.

(95 CI: 73.4–90.0 and 65.7–84.0%, respectively). The surgical procedure (segmentectomy or wedge resection), age and co-morbidities were prognostic factors in overall survival. In Cox's multivariate analysis, wedge resection was the only unfavourable prognostic factor for overall survival ($P = 0.02$, HR = 2.8) (Fig. 6A). In disease-free survival, an unfavourable prognostic factor was not apparent (wedge resection: $P = 0.09$, Fig. 6B).

DISCUSSION

In the present study, excellent results were seen for patients who had limited resections for clinical stage IA NSCLC and who were diagnosed as able to tolerate lobectomy and were 75 years or

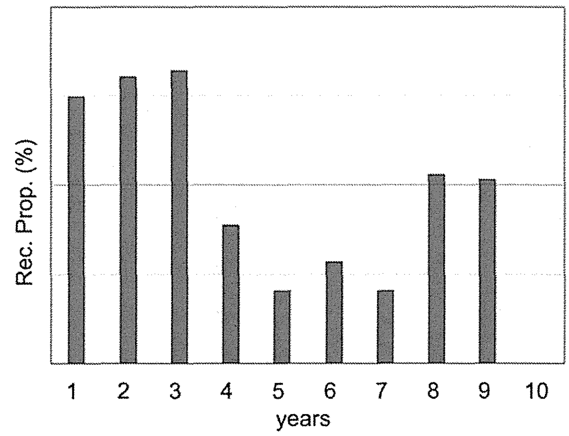

Figure 3: Recurrence proportion for 10 years, representing the ratio of the number of patients with recurrence and the number of patients at risk every year.

younger at the time of operation. This study predominantly included GGNs and part-solid nodules. Unfortunately, we did not know the precise ratio of GGNs; even in radiological invasive carcinomas, many part-solid nodules were included. The 5-year overall survival was 94.0% and was not inferior to the reported survival of lobectomy patients of cStage IA adenocarcinoma [11]. In early-stage NSCLC, curative surgical resection has been considered to be the most important determinant of prognosis [1, 2]. Lobectomy with lymph node dissection remains the standard procedure in tolerable cases. Ginsberg and Rubinstein [3] compared the results of lobectomy and limited resection for stage I lung cancer in 1995. To date, this has been the only randomized trial on limited resection versus lobectomy for lung cancer. Higher death and local recurrence rates were demonstrated in patients undergoing limited resection [3]. However, excellent survival data of limited resection have been reported in patients with small-sized NSCLC [4–8]. Maximum tumour diameter has been discussed as a criterion for an acceptable limited resection. A size of <2 cm was proposed in some reports [4–6], while another [6] suggested <1 cm as the criterion to obtain survival in limited resections equivalent to lobectomy [8]. In this study, the maximum tumour diameter was not a significant prognostic factor. However, pT2 and T3 diseases showed significantly unfavourable prognosis. We could not ignore the maximum tumour diameter.

Stage I lung cancer consists of a heterogeneous population and may include latent systemic diseases. A 'very early' lung carcinoma is a good candidate for limited resection if it can be defined by CT images or other biological markers. Recently, the definitions of adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA) were introduced by the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society [15]. Pathologically, AIS and MIA are 'very early' and may be good candidates for limited resection. Radiological findings that define these early cancers have been proposed. The JCOG conducted a prospective radiological study of thin-section CT to identify criteria that predict pathological noninvasiveness in clinical stage IA lung cancer (JCOG0201) [10, 11]. A C/T ratio of 0.25 or less among cT1a (≤ 2.0 cm) was shown to be a good radiological indication of histologically non-invasive adenocarcinoma [10, 11]. In the present study, the group with a C/T ratio of 0.25 or less showed better prognosis and this C/T ≤ 0.25 seemed to be a good radiological indicator of

Table 2: Multivariate analysis of factors associated with survival and proportion

Factor	HR[a]	Lower 95% CI[b]	Upper 95% CI	P***
Overall survival				
pN+	7.07	3.63	13.8	<0.001
Male	2.80	1.87	4.20	<0.001
IP +	2.23	1.06	4.69	<0.001
Age ≥ 65	2.05	1.41	2.99	<0.001
pT2a-3	1.86	1.15	3.02	0.011
C/T > 0.25	1.77	1.14	2.74	0.011
Comorbidity +	1.64	1.15	2.32	0.006
Disease-free survival				
pN+	6.71	3.65	12.4	<0.001
Male	2.59	1.85	3.63	<0.001
C/T > 0.25	2.62	1.76	3.90	<0.001
pT2a-3	2.11	1.40	3.17	<0.001
Low %VC	2.03	1.28	3.21	0.026
IP+	2.02	1.03	3.97	0.041
Age ≥ 65	1.79	1.31	2.46	<0.001
Comorbidity +	1.68	1.25	2.25	<0.001
Recurrence-free proportion				
pN+	6.95	3.54	13.7	<0.001
C/T > 0.25	4.72	2.52	8.85	<0.001
IP +	2.78	1.33	5.83	<0.001
pT2a-3	2.78	1.73	4.49	<0.001
Male	2.35	1.54	3.58	<0.001
Low %VC	2.30	1.31	4.05	0.004
Comorbidity +	1.72	1.19	2.50	0.004
Free proportion from death by other causes				
Male	3.16	1.79	5.57	<0.001
Age ≥ 65	3.01	1.71	5.32	<0.001
SqCC	2.19	1.07	4.48	0.032

C/T: the ratio of the consolidation (C) to the maximal tumour diameter (T); IP+: comorbidity of interstitial pneumonia; low %VC: %vital capacity <80%; HR: hazard ratio; CI: confidence interval.
****P* is shown as $P < 0.001$ if the actual *P*-value was <0.001.

noninvasive cancer. Lung cancers that met this criterion rarely recurred after limited resection. The recurrence-free proportion was 98.5% at 5 years (95 CI: 97.3–99.2%), ($P < 0.0001$, Fig. 5C). A prospective phase II study of limited resection for peripheral early lung cancer using the same criteria with thoracic thin-section CT was conducted (JCOG0804/WJOG4507L). Although patient enrolment ended in 2011, more time will be needed before the results of this trial are available. In practice, limited resection for lung cancer has been widely used, especially as an alternative for patients who will not tolerate lobectomy for functional or other reasons [12, 13]. To be sure, there are tumours that are cured by limited resection, especially when the tumour is small and non-invasive. With the demonstration of the validity of radiological criteria of non-invasive adenocarcinoma, more small GGNs are found by high-resolution CT, and limited resection has been increasing in clinical practice. The present study was conducted in order to investigate the best available long-term results to date of limited resections for lung cancers. Although the study was retrospective and not directly comparable with the prospective study of lobectomy reported for early lung cancer [11], limited resection did not appear to be inferior to lobectomy. In our study, patient fitness for lobectomy was diagnosed by the pulmonary function test result and comorbidities, and the diagnostic criteria may have differed from institution to institution. Even with these limitations, the results on a large cohort of 1737 patients offer a reliable indication that limited resection for selected clinical stage I NSCLC patients is a satisfactory curative surgical procedure. The 95% CIs were extremely narrow. The overall survival in 1737 patients was 94.0% at 5 years (95 CI: 92.6–95.1%), and disease-free survival was 91.1% at 5 years (95 CI: 89.5–92.4%).

Overall, we found that selection of patients by Japanese surgeons was reasonable. Most of the patients were without IP (98.2%) and had none or only one comorbidity (94.1%). The lung cancers presented were cT1aN0M0 diseases in 90.7%. The recurrence rate was low in such patients. However, the prognosis of the patients who had recurrences was unfavourable. The overall survival in all 122 patients with recurrence was 57.4% at 5 years. The median survival time after recurrence of these patients was 34.7 months. This suggests that the recurring lung cancer had high malignant potential and was different from those that did not recur. CT finding of C/T ratio > 0.25 was a significant prognostic factor for recurrence (HR: 4.72, $P < 0.001$). The recurrence was diagnosed mostly within 3 years of the operation and the second peak was recognized around 8–9 years postoperatively. Nakao *et al.* [13] reported the necessity of long-term follow-up for the patients who underwent limited resections for lung cancers with ground-glass opacity nodules which usually grow very slowly.

A subclass analysis was performed on 1538 patients with cT1aN0M0 disease excluding those with multiple lesions or with IP, in order to compare our results with those of JCOG0201 [11]. In that study, the 5-year overall and recurrence-free survivals in 289

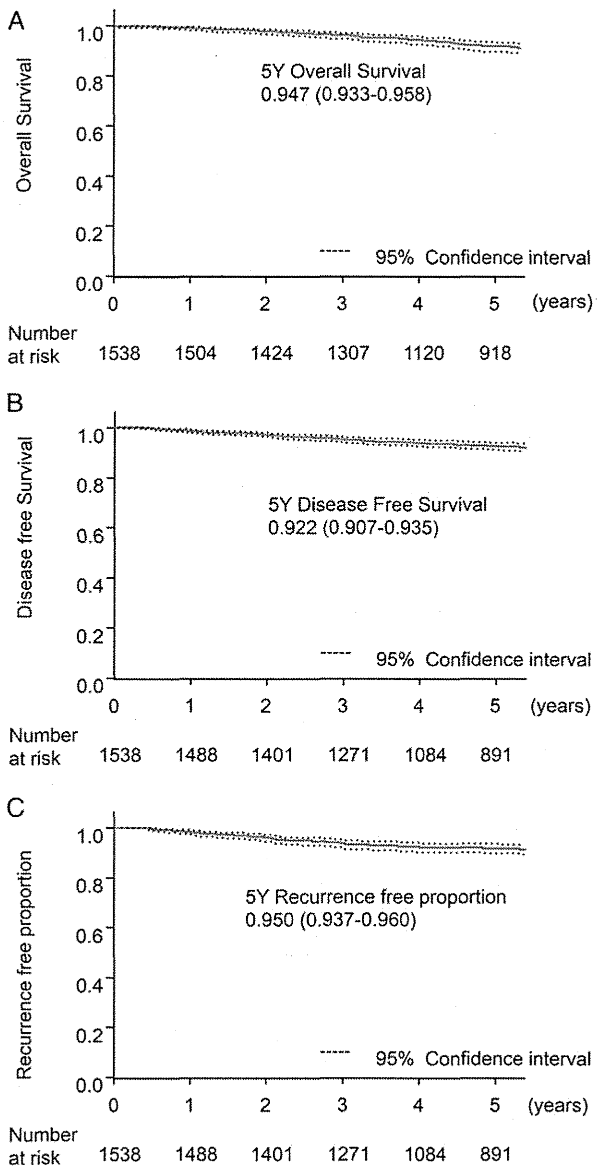

Figure 4: Survival and recurrence data of the 1538 cT1aN0M0 patients with a single lesion and without comorbidity of interstitial pneumonia. **(A)** Five-year overall survival. **(B)** Five-year disease-free survival. **(C)** Five-year recurrence-free proportion.

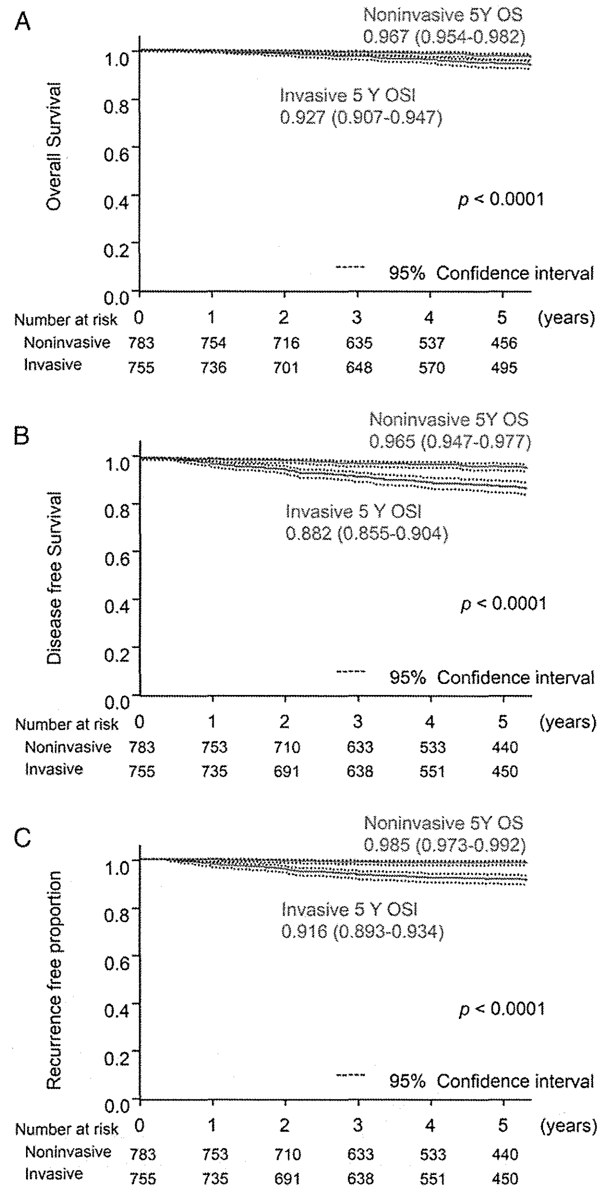

Figure 5: Radiological findings and prognosis of the 1538 cT1aN0M0 patients with a single lesion and without comorbidity of interstitial pneumonia. Comparison between radiological invasive carcinomas ($n = 755$) and radiological non-invasive carcinomas ($n = 783$). **(A)** Five-year overall survival. **(B)** Five-year disease-free survival. **(C)** Five-year recurrence-free proportion.

patients who underwent lobectomy for cT1aN0M0 adenocarcinoma were 93.0 and 88.9%, respectively [11]. Both the 5-year overall and recurrence-free survival rates in 35 patients of C/T ratio ≤0.25 were 97.1% [11]. These data are very similar to our data of limited resections: 94.7 (95 CI: 93.3–95.8%) and 92.2 (95 CI: 90.7–93.5%) for 5-year overall and recurrence-free survivals, respectively, in the JCOG0201; 96.7 (95 CI: 95.4–98.2%) and 96.5 (95 CI: 94.7–97.7%) for the 5-year overall and recurrence-free survivals, respectively, in the 783 patients with tumours of C/T ratio ≤0.25 in our cohort. The lower limit of the 95% CI for the overall survival rate at 5 years in our limited resection patients is higher than that of the lobectomy patients in the JCOG 0201 study. Although it is not possible to directly compare the results of the two studies, these data suggest that the efficacy of limited resection for selected NSCLC patients is not inferior to that of lobectomy. We eagerly await the results of the prospective study (JCOG0804/WJOG4507L), which may finally demonstrate the efficacy of limited resections.

Finally, a subclass analysis of 100 SqCC patients was performed. Unfavourable prognostic factor in overall survival was wedge resection. There was no difference between cT1aN0M0 disease and cT1bN0M0 disease. Prognosis of the SqCC patients who underwent segmentectomy was superior to those who underwent wedge resection. This result was different from the data of all 1737 patients or 1538 patients with cT1aN0M0 adenocarcinoma. Differences in procedures of limited resection should be partly dependent on the differences of pathology. The result appears to suggest that for SqCC nodules segmentectomy or larger resection should be selected.

It should be noted that segmentectomy in this study did not provide better protection from recurrence. There were no

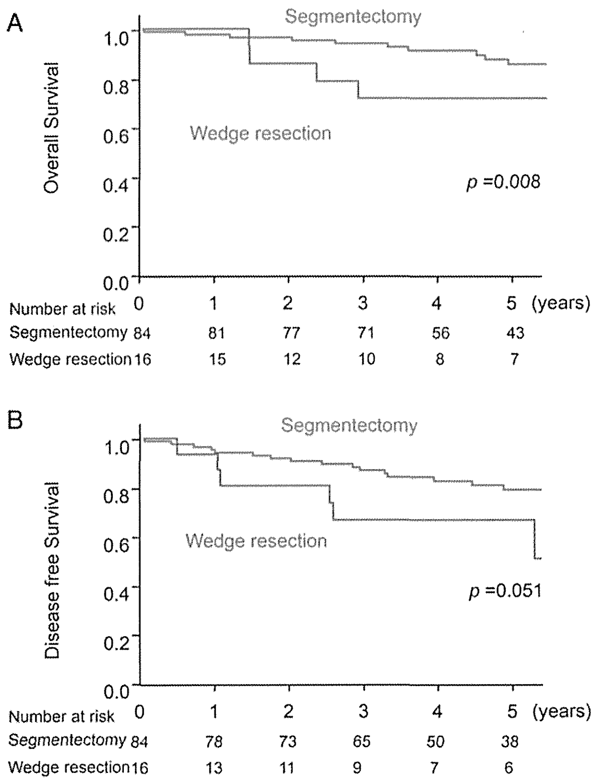

Figure 6: Prognosis of the 100 patients with s cT1N0M0 squamous cell carcinoma disease. (**A**) Five-year overall survival. (**B**) Five-year disease-free survival.

prognostic differences between segmentectomy and wedge resection in overall survival. In small-sized NSCLCs, segmentectomy has been considered an alternative to lobectomy [4, 5] and to be superior to wedge resections in prognosis of early-stage NSCLCs [4, 5, 16–18]. Smith *et al.* [18] recently reported that overall and lung cancer-specific survivals were significantly better among patients who underwent segmentectomy than with wedge resection in a large number of stage IA NSCLC patients registered in the SEER registry. In the present study, segmentectomy was selected more frequently than wedge resection in larger tumours ($P < 0.0001$) and a candidate of radiological invasive tumours (C/T > 0.25) ($P < 0.0001$). As a result of a subgroup analysis, we found the operative procedure-influenced survival in tumours with SqCC pathology. Segmentectomy should be recommended for SqCC even for small-sized nodules.

We emphasize that our data do not recommend limited resection for solid carcinomas, although the prognosis of limited resection for tumours with C/T ≤ 0.25 was excellent. Those carcinomas with a C/T ratio >0.25 include pure solid nodules which may be aggressive and part-solid nodules which may be less aggressive. We reserve our recommendation of limited resection only for carcinomas with C/T ≤ 0.25.

In conclusion, using the data of 1963 patients, excellent results have been shown of limited resections for small-sized NSCLC in patients who would tolerate lobectomy. The prognosis of these patients was no worse than that of the similarly carefully selected lobectomy patients. The carcinomas with C/T ≤ 0.25 are good candidates for limited resection.

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Conflict of interest: none declared.

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

A Retrospective Analysis of 2657 Patients

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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.

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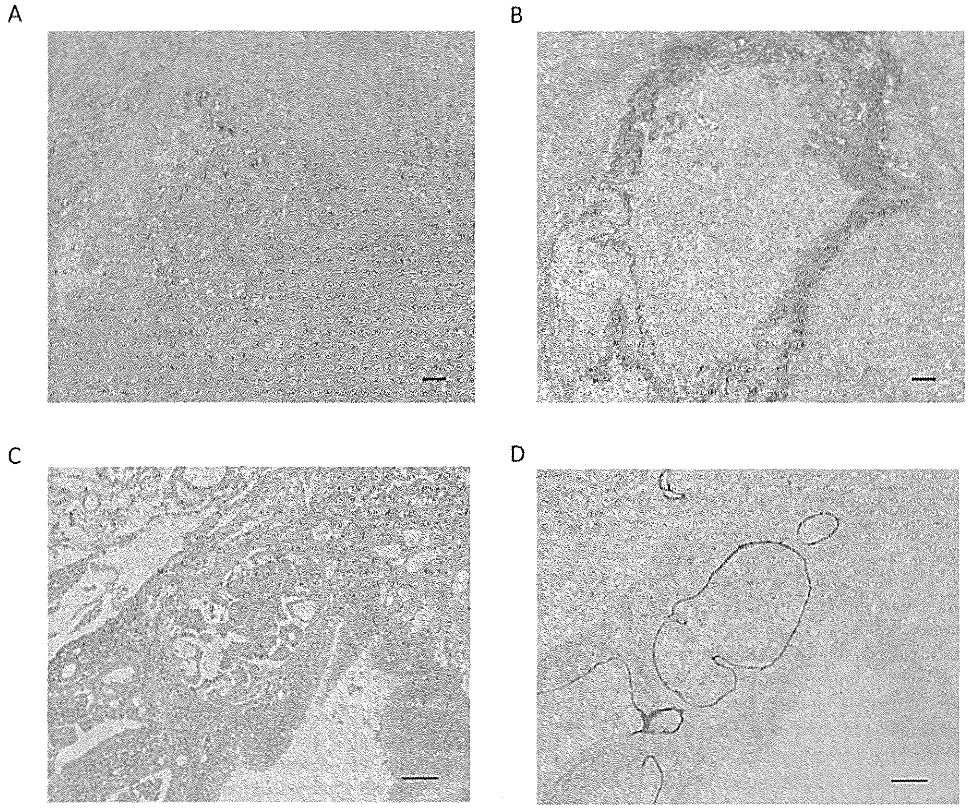

FIGURE 1. Microscopic findings of BVI (A and B) and LVI (C and D). A, BVI with H&E staining; B, BVI with Victoria blue–van Gieson staining; C, LVI with H&E staining; and D, LVI with D2-40 staining. Scale bar, 100 μm.

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