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Isolation of miRNAs that target EGFR mRNA in human lung cancer

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ABSTRACT

Lung cancer, predominantly non-small cell lung cancer (NSCLC), remains the leading cause of cancer-related deaths worldwide. Although epidermal growth factor receptor (EGFR) signaling is important and well studied with respect to NSCLC progression, little is known about how miRNAs mediate EGFR signaling to modulate tumorigenesis. To identify miRNAs that target EGFR, we performed a bioinformatics analysis and found that miR-542-5p down-regulates *EGFR* mRNA and protein expression in human lung cancer cells (H3255, A549, Hcc827). We observed increases in EGFR association with Ago2 in miR-542-5p-transfected cells. Interestingly, we observed an inverse correlation of miR-542-5p expression and EGFR protein levels in human lung cancer tissue samples, suggesting that miR-542-5p directly targets EGFR mRNA. Furthermore, we found that miR-542-5p inhibited the growth of human lung cancer cells. Our findings suggest that miR-542-5p may act as an important modulator of EGFR-mediated oncogenesis, with potential applications as a novel therapeutic target in lung cancer.

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1. Introduction

Lung cancer is the most common cancer and the leading cause of cancer-related death worldwide [1]. The epidermal growth factor receptor (EGFR) signaling network plays a central role in the growth and maintenance of epithelial tissues, and EGFR is overexpressed or mutated in most non-small cell lung cancer (NSCLC) cases [2]. Consequently, the EGFR and its downstream signaling effectors are major targets for new therapeutics such as monoclonal antibodies and tyrosine kinase inhibitors [3]. However, the clinical responses of tumors to existing anti-EGFR agents are often limited, and thus a major research focus is the development of novel approaches to block EGFR expression and signaling [4].

MicroRNAs (miRNA) belong to a class of endogenously-expressed, non-coding small RNAs of approximately 22 nucleotides. These small RNAs influence gene regulation by pairing to protein-coding mRNAs to repress their expression via decreased translational efficiency and/or mRNA levels [5]. Growing evidence suggests that dysregulation of miRNA expression contributes to a wide variety of human cancers, including lung cancers [6–11]. Recently, miRNAs have been demonstrated to be diagnostic and prognostic markers in leukemia, lung cancer, and colon cancer [12]. miRNAs may also represent therapeutic targets in human cancers [13]. Interestingly, it has been reported that miR-7 has the ability to coordinately regulate EGFR signaling in multiple human cancer cell types [14]. A miRNA that regulates EGFR may have therapeutic potential against lung cancer.

Computational approaches to miRNA target prediction have used criteria such as sequence complementarity between target mRNAs and a "seed" region within the miRNA, and conservation of predicted miRNA-binding sites across 3′-UTRs from multiple species [15]. Recently, additional features that determine target site functionality have been identified [11,16]. However, the imperfect complementarity of miRNA and target sequences means that identification and functional validation of authentic miRNA targets remains a major challenge.

In the present study we investigate miRNAs that might target EGFR mRNA using computational approaches and identified miR-542-5p as a direct regulator of EGFR mRNA in cancer cells. Furthermore, we showed that miR-542-5p suppressed proliferation of lung cancer cells. Identifying miRNA regulators of EGFR may contribute to the development of novel therapeutics.

2. Materials and methods

2.1. Cell culture and transfection

The HeLa human cervical cancer cell line and the A549 human lung cancer cell line were purchased from the American Type Culture Collection. Cells were cultured according to ATCC instructions. MicroRNAs used in this study were as follows: has-miR-7

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sense (5′-UGGAAGACUAGUGAUUUUGUUGU-3′) and antisense (5′-AACAAGUCACAGCCGGCCUCA-3′); hsa-miR-541 sense (5′-AAAGG AUUCUGCUGUCGGUCCCACU-3′) and antisense (5′-UGGUGGGCAC AGAAUCUGGACU-3′); has-miR-542-5p sense (5′-UCGGGGAUCAU CAUGUCAUGAGA-3′) and antisense (5′-ugugacagauugauaacugaaa-3′) miR-nontarget control miRNA sense (5′-AUCCGCGCGAUAG CACGUAUU-3′) and antisense (5′-UACGUACUAUCGCGCGGAUUU-3′). Oligonucleotides were individually transfected into cells using HiPerFect reagent (Qiagen) according to the manufacturer's instructions.

2.2. Western blot analysis

Protein samples were suspended in sodium dodecyl sulfate loading buffer. After boiling, equal amounts (20 μg) of the protein samples were run on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to Immobilon membranes (Millipore, Bedford, MA) by semi-dry blotting. The membranes were probed with antibody for EGFR (sc-71033; Santa Cruz Biotechnology) using standard techniques. The signals were visualized by ECL Plus Western blotting detection system (GE Health Care) and detected with LAS-3000 mini (Fujifilm).

2.3. RNA isolation and quantitative RT-PCR

RNAs were isolated from miR-transfected A549 cells using Isogen reagent (Nippon Gene) according to the manufacturer's instructions. miRNA levels were quantified using TaqMan MicroRNA Assays (Applied Biosystems). miRNA levels were normalized based on has-miR-16 levels. Complementary DNA was synthesized using SuperScriptII and Random Hexamers (Invitrogen). Quantitative PCR analysis was run on a Stratagene MX3000P thermocycler and analyzed with MxPro (Stratagene). The EGFR primers used in this study were as follows: forward primer, 5′-GTGACCGTTTGG-GAGTTGATGA-3′ and reverse primer, 5′-GGCTGAGGGAGGCGTTCT C-3′.

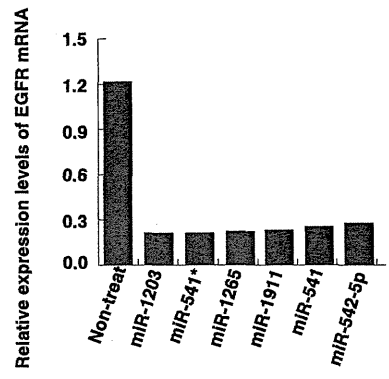
2.4. Immunohistochemistry and in situ hybridization

Human lung cancer tissues for histologic studies were obtained from Tokyo Medical University Hospital. This study was approved

A

Luciferase GL3-6FAM	Has-mir-663b	Has-miR-1908	Has-miR-452	Has-miR-671-5P	Has-miR-1538	Has-miR-150*	Has-miR-616	Has-miR-1203	Has-miR-575	Lamin A/C
Has-miR-7	Has-miR-1205	Has-miR-1226*	Has-miR-146b-3p	Has-miR-1301	Has-miR-1911	Has-miR-541*	Has-miR-637	Has-miR-1265	Has-miR-1237	Has-miR-517*
Has-miR-24	Has-miR-1207-5p	Has-miR-1914*	Has-miR-432	Has-miR-770-5p	Has-miR-21*	Has-miR-198	Has-miR-663	Has-miR-612	Has-miR-377*	Has-miR-1228*
Has-miR-188-3p	Has-miR-1244	Has-miR-214	Has-miR-520a-5p	Has-miR-760	Has-miR-92a-2*	Has-miR-139-3p	Has-miR-30b*	Has-miR-550	Has-miR-941	Has-miR-1911*
Has-miR-296-3p	Has-miR-1254	Has-miR-193a-5p	Has-miR-526b	Has-miR-934	Has-miR-23b*	Has-miR-330-5p	Has-miR-541	Has-miR-30a*	Has-miR-542-5p	Has-miR-453
Has-miR-423-5p	Has-miR-1269	Has-miR-370	Has-miR-500	Has-miR-1200	Has-miR-130a*	Has-miR-485-5p	Has-miR-665	Has-miR-93*	Has-miR-624*	Has-miR-557
Has-miR-503	Has-miR-1292	Has-miR-378	Has-miR-509-3p	Has-miR-1250	Has-miR-143*	Has-miR-520a-5p	Has-miR-1915	Has-miR-185*	Has-miR-886-5p	Luciferase GL3
eGFP	Has-miR-1471	Has-miR-422a	Has-miR-532-5p	Has-miR-1268	Has-miR-149*	Has-miR-525-5p	Has-miR-1184	Has-miR-194*	Has-miR-412	Negative contorol

B



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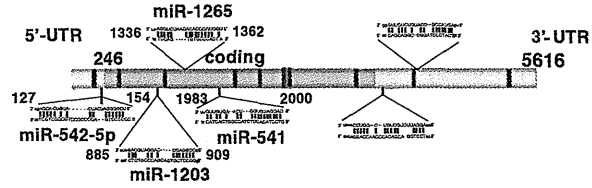


Fig. 1. Identification of miRNAs that target EGFR using 96-well plate transfections. (A) Candidate miRNAs targeting the EGFR sequence on a 96-well plate. (B) Down-regulation of EGFR mRNA by synthetic miRNA. Real-time PCR showed that synthetic miR-1203, miR-541*, miR-1265, miR-1911, miR-541, and miR-542-5p suppress EGFR mRNA in HeLa cells. (C) The predicted binding sites for miR-1203, miR-541*, miR-1265, miR-1911, miR-541, and miR-542-5p in the EGFR mRNA are indicated schematically.

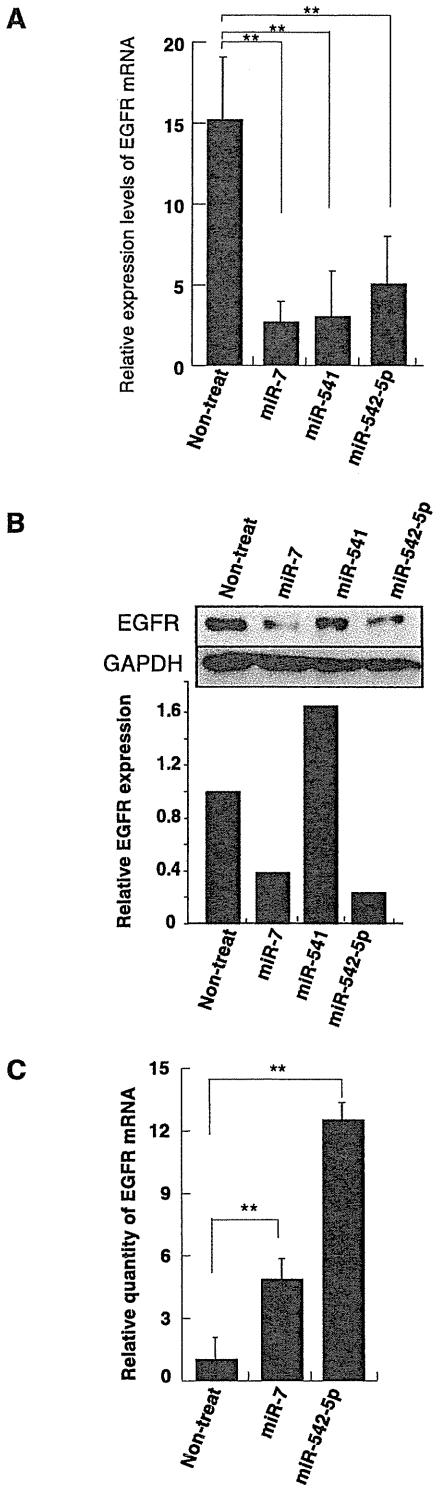


Fig. 2. EGFR is a direct target of miR-542-5p in lung cancer cells. (A) Quantitative analysis of EGFR mRNA by real-time PCR. EGFR mRNA significantly decreased upon transfection of synthetic miR-7, miR-541, and miR-542-5p in A549 cells. *Bars*, mean ± SD. $^{**}P < 0.02$ (B) Regulation of EGFR protein expression by synthetic miR-7, miR-541, and miR-542-5p. A representative western blot analysis of total cell extracts from A549 cells transfected with synthetic miR-7, miR-541, and miR-542-5p is shown in (B). GAPDH was used as a loading control. Densitometry ratios of EGFR to GAPDH were calculated and recorded. (C) Using an anti-Ago2 antibody, we performed RNA co-immunoprecipitation from A549 cells transfected with synthetic miR-7 and miR-542-5p. Reverse-transcribed RNA was PCR-amplified using primers specific for the EGFR mRNA. The averages of three independent experiments are shown. *Bars*, mean ± SD. $^{**}P < 0.02$.

by the institutional review board of Tokyo Medical University, and all patients provided written informed consent.

Immunohistochemical assays were performed on formalin-fixed, paraffin-embedded sections with the Ventana HX System Benchmark (Ventana Medical Systems). An anti-EGFR monoclonal antibody (DAK-H1-WT, DAKO) was applied at a dilution of 1:500. miR-542-5p expression in human lung cancer specimens was detected by *in situ* hybridization with miRCURY LNA probe for miR-542-5p (Exigon) as described previously [17].

2.5. In vitro proliferation assays

We evaluated the effects of miR-7 and miR-542-5p on A549 cell growth using the MTT metabolic growth assay kit (Cell Count Reagent SF, Nacalai Tesque). After transfection with miR-7 or miR-542-5p, cell numbers were assessed by MTT assay 72 h after transfection according to the manufacturer's instructions. Briefly, reagents were added to each well and incubated at 37 °C for 4 h. The reduction of MTT by living cells into a formazan product was visualized using a multiwell scanning spectrophotometer at 450 nm.

2.6. Co-immunoprecipitation

miRNA-542-5p and Ago2 co-immunoprecipitation experiments were performed using Ago2 antibody (Wako Pure Chemical Industries, Tokyo, Japan) as described previously [17]. Total RNA was isolated from the precipitates using TRIzol reagent. Reverse transcription-PCR was performed as described. Parallel immunoprecipitation using rabbit IgG served as a control.

2.7. Statistical analysis

Differences were statistically evaluated using one-way ANOVA followed by Fisher's protected least significant difference test. P values < 0.05 were considered statistically significant.

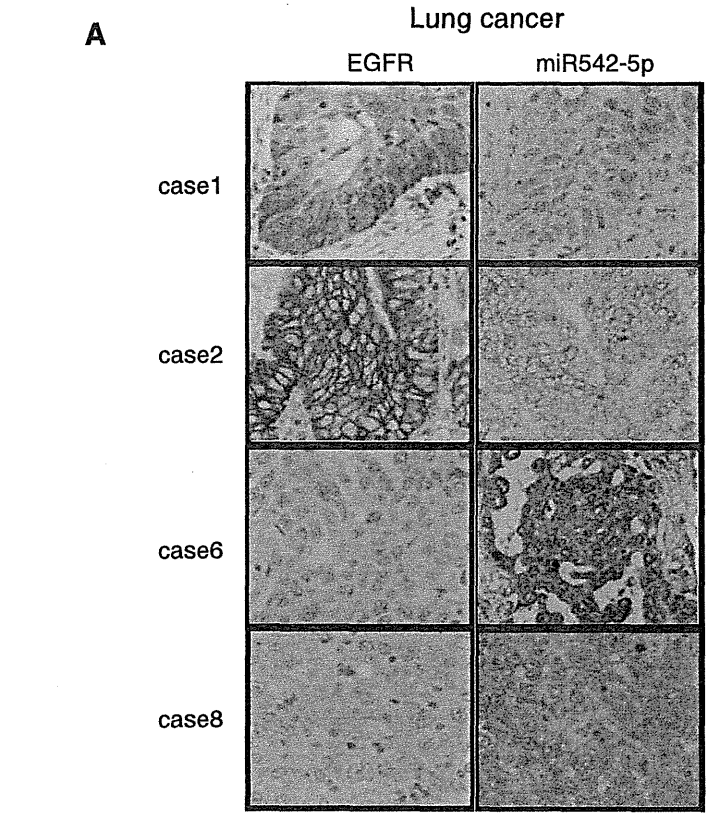
3. Results

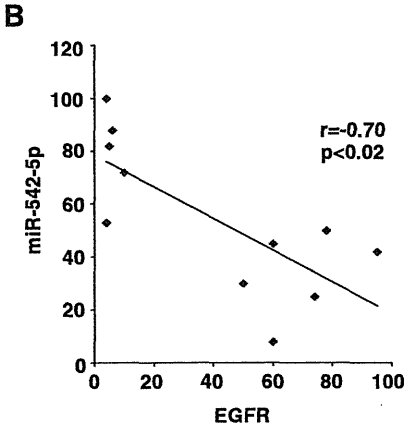
3.1. Identification of EGFR target miRNA

First, we used the miRanda system to select candidate miRNAs that might regulate EGFR (NM_201281.1, NM201282.1, NM201283.1, NM201284.1 and NM005228.3). In this analysis, we evaluated the full-length EGFR gene, including the 5′-UTR. We selected a total of 413 candidate miRNAs that could potentially target EGFR (Supplement 1). We narrowed the list of miRNAs based on pairing scores and energies of sequence and synthesized 83 miRNAs (Fig. 1A). We next transfected HeLa cells in 96-well plates with the miRNAs and determined the expression of EGFR mRNA by qRT-PCR analysis. miR-1237, -1203, -541*, -1265, -1911, -541, and -542-5p down-regulated EGFR mRNA in HeLa cells (Fig. 1B, C).

3.2. miR-542-5p directly targets EGFR in lung cancer cell lines

Next, we determined whether the above miRNAs down-regulate EGFR in the A549 lung cancer cell line. We transfected miR-1237, -1203, -541*, -1265, -1911, -541, and -542-5p as well as miR-7, which has been reported to down-regulate EGFR mRNA and protein expression in lung cancer cell lines [14]. In A549 cells, RT-PCR analysis showed that miR-541, miR-542-5p, and miR-7 down-regulate EGFR mRNA (Fig. 2A); however, we could not confirm down-regulation of EGFR mRNA by miR-1237, -1203, -541*, -1265, or -1911.





C

	EGFR	miR-542-5p
case1	82	5
case2	72	10
case3	25	74
case4	50	78
case5	8	60
case6	42	95
case7	53	4
case8	45	60
case9	100	4
case10	88	6
case11	30	50

Fig. 3. EGFR expression in human lung cancer cells is inversely related to miR-542-5p expression. (A) We performed *in situ* hybridization for miR-542-5p in concert with immunohistochemical analysis for EGFR. Blue staining represents miRNA positivity, while brown staining represents EGFR positivity. (B) The inverse correlation between miR-542-5p expression and EGFR expression in lung adenocarcinomas is graphically depicted. The mean miR-542-5p and EGFR staining scores were calculated as described [26]. *Bars*, mean ± SD. *$P < 0.02$, ANOVA followed by Tukey–Kramer test. (C) Summary of EGFR and miR-542-5p staining data.

We next investigated whether miR-541 and miR-542-5p suppressed EGFR protein expression. Interestingly, we observed that miR-7 and miR-542-5p down-regulated EGFR protein, whereas miR-541 did not affect EGFR protein levels. These data indicate that miR-542-5p directly suppresses translation of EGFR mRNA. Next, we performed immunoprecipitation assays using an anti-Ago2 antibody. Ago2 is an essential mediator of miRNA-binding to RNA-induced silencing complexes. We therefore analyzed EGFR mRNA levels in Ago2 immunoprecipitates from lysates of HeLa cells transfected with either miR-7 or miR-542-5p. After normalizing EGFR mRNA levels to β-actin mRNA levels in IP samples, we observed increases in EGFR mRNA association with Ago2 in miR-7 and miR-542-5p-transfected cells (Fig. 2C). Together, these data indicate that EGFR mRNA is a direct target of miR-542-5p.

3.3. EGFR is up-regulated in human lung cancer in association with miR-542-5p expression

To investigate the connection between EGFR expression and *miR*-542-5p in lung cancer, we performed immunohistochemical

analysis using anti-EGFR antibodies and *in situ* hybridization using LNA-modified probes specific for *miR*-542-5p in a series of clinical lung cancer samples. Positive staining for *miR*-542-5p was observed in the cytoplasm within these specimens (Fig. 3A). We also detected positive cytoplasmic and membrane staining for EGFR (Fig. 3A). The majority of cases with low *miR*-542-5p staining exhibited strong expression of EGFR protein (Fig. 3B), while most tissues demonstrating high staining for *miR*-542-5p showed low EGFR protein expression. These data demonstrate an inverse correlation of *miR*-542-5p expression with EGFR protein levels *in vivo* ($r = -0.7$, $P < 0.02$, by Spearman's correlation coefficient by rank test), and suggest that dysregulation of *miR*-542-5p is involved in lung carcinogenesis and tumor progression by targeting EGFR.

3.4. Effects of miR-542-5p on a human lung cancer cell line

The EGFR oncoprotein plays a pivotal role in the proliferation of lung cancer cells [18]. Therefore, we investigated whether miR-542-5p would affect the proliferation of the A549 human lung cancer cell line. We transiently transfected either anti-miR-542-5p or miR-7 antagomir into these cells and assessed cell number by MTT assay three days after transfection. Cells transfected with anti-542-5p or miR-7 antagomir exhibited lower proliferative rates than cells transfected with control LNA (Fig. 4: $P < 0.05$). We confirmed the levels of miR-542-5p and miR-7 in cells by quantitative real-time PCR (data not shown). These results suggest that *miR*-542-5p may serve as a molecular target for novel anticancer drugs.

4. Discussion

In the present study, we have demonstrated that miR-542-5p can regulate the expression of EGFR. Furthermore, miR-542-5p has functional effects in cancer cell lines that include reducing cell growth and viability.

Recently, it has been reported that miR-7 down-regulates EGFR mRNA and protein expression in cancer cell lines (lung, breast, and glioblastoma), inducing cell cycle arrest and cell death [14]. We confirmed that miR-7 suppresses EGFR mRNA and protein levels and found that miR-542-5p does as well. Interestingly, miR-542-5p more strongly suppressed cell proliferation than miR-7 (Fig. 4). One possibility for this difference is that miR-7 binds to the 3′-UTR of the EGFR mRNA whereas miR-542-5p binds to the 5′-UTR. Recently, it has become apparent that miRNAs can target sites in 5′-UTRs, and that interactions of miRNAs with gene promoters can regulate gene activity at the transcriptional level [19]. miR-7 has the ability to coordinately down-regulate the expression of multiple members of the EGFR signaling cascade [14]. It is possible that these miRNAs have different potential to down-regulate the expression of multiple members of the EGFR signaling cascade.

Selective inhibitors of EGFR tyrosine kinase activity (EGFR-TKI; i.e., gefitinib and erlotinib) prevent binding of ATP to the ATP-binding pocket of EGFR in a competitive manner, resulting in the loss of catalytic activity [20,21]. Interestingly, it has been reported that the effects of EGFR-TKI are correlated with activating somatic mutations in the epidermal growth factor receptor [22–24]. In contrast, miRNA-542-5p inhibits EGFR by a different mechanism – down-regulating its levels by binding to the 5′-UTR of the EGFR mRNA. Therefore, the suppression of EGFR by miRNA-542-5p should not be affected by mutations in the coding sequence. Agents such as miR-542-5p that down-regulate expression of EGFR as well as some of its signaling effectors may have significant therapeutic potential in a range of human cancer types.

It has recently been reported that miR-543-5p plays a tumor suppressor role in neuroblastoma cells [25], but the proposed target gene was nuclear. In this study, we showed that miR-542-5p

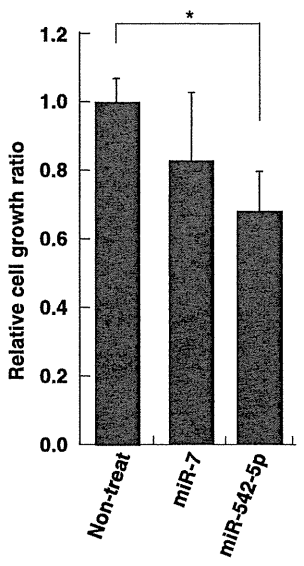


Fig. 4. miR-542-5p modulates the proliferation of human lung cancer cell lines. We determined the number of A549 cells 72 h after transfection with the amiR-7 or miR-542-5p by MTT assay. *Bars*, mean ± SD. (*$P < 0.05$).

overexpression in lung cancer cells decreased cell numbers compared with non-treated cells (Fig. 4). These data indicate that miR-542-5p is a potential novel therapeutic target in lung cancer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.008.

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Pathological Vascular Invasion and Tumor Differentiation Predict Cancer Recurrence in Stage IA Non–Small-Cell Lung Cancer After Complete Surgical Resection

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Introduction: The appropriate therapeutic strategy and postoperative management for patients with stage IA non–small-cell lung cancer (NSCLC) still remain a matter of debate because of the prognostic heterogeneity of this population, including the risk of cancer recurrence. The objective of the current study was to identify the clinicopathological factors that affect overall prognosis and cancer recurrence of stage IA NSCLC.

Methods: We reviewed the data of 532 patients in whom complete resection of stage IA NSCLC had been performed. Overall survival and recurrence-free proportion (RFP) were estimated using the Kaplan–Meier method. RFP was estimated from the date of the primary tumor resection to the date of the first recurrence or last follow-up. We performed univariate and multivariate analyses to determine the independent prognostic factors.

Results: On multivariate analyses, three variables were shown to be independently significant recurrence risk factors: histological differentiation (hazard ratio [HR] = 1.925), blood-vessel invasion (HR = 1.712), and lymph-vessel invasion (HR = 1.751). On subgroup analyses combining these risk factors, the 5-year RFP was 91.3% for patients with no risk factors, 79.5% for those with either poorly differentiated carcinoma or vascular invasion, ($p < 0.001$ for both), and 62.9% for those with both poorly differentiated carcinoma and vascular invasion ($p = 0.068$).

Conclusion: These results indicated that vascular invasion and tumor differentiation have a significant impact on the prediction of cancer recurrence in patients with stage IA NSCLC. Patients with these predictive factors of recurrence may be good candidates for adjuvant chemotherapy.

Key Words: Prognostic factor, Non–small-cell lung cancer, Recurrence, Stage IA, Vascular invasion, Tumor differentiation.

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The tumor, node, metastasis (TNM) staging system for non–small-cell lung cancer (NSCLC) is currently the best confirmed predictor of survival and guide for treatment. NSCLC patients with pathologic stage IA disease have the best chance of survival, and resection is standard in such cases. However, even after curative resection, the 5-year survival rate is between 80% and 87% in pathologic stage IA patients as shown in large-scale Japanese lung cancer studies,[1–3] and recent data from the lung cancer staging project of the International Association for the Study of Lung Cancer revealed a 5-year survival rate of 73% for pathological stage IA patients.[4] Therefore, up to 10% of patients with stage IA NSCLC have recurrence after surgery, even in cases with early-stage disease.

Many studies of resected specimens have been performed to determine various clinicopathological prognostic factors other than the pathologic stage for these patients, such as sex, age,[5] smoking history,[6] serum level of carcinoembryonic antigen (CEA),[7] extent of operation,[5] tumor size, vascular invasion,[7–18] and the grade of differentiation of the tumor.[14,17,19] Patients, including those with stage IA NSCLC, who have such factors may be good candidates for receiving systemic therapy such as adjuvant chemotherapy. The objective of the present study was to identify the clinicopathological factors that affect overall prognosis and cancer recurrence of stage IA NSCLC in a single institution.

PATIENTS AND METHODS

Patients

From January 1990 to December 2007, a total of 1973 patients underwent complete pulmonary resection for NSCLC at our hospital. Complete resection was defined as cancer-free surgical margins both grossly and histologically. All the patients underwent radical surgical resection and systematic mediastinal lymph node dissection. Of these, 674 patients with consecutive pathologic stage IA NSCLC were identified in our departmental database. The number of resected lymph nodes ranged from one to 49, with a mean of 15. We excluded 142 patients who had undergone preoperative chemotherapy

or radiotherapy (n = 17), postoperative treatment including chemotherapy or chemoradiotherapy (n = 105), and those who had low-grade malignant tumors including carcinoids, mucoepidermoid carcinomas, or adenoid cystic carcinomas (n = 20). The remaining 532 patients comprised the subjects of this study.

Preoperative evaluation included physical examination, chest radiography, computed tomography (CT) of the chest and abdomen, bone scintigraphy, blood examination, and since the early 2000s, positron-emission tomography (PET) scan (recently performed as integrated PET-CT scan). Most patients were postoperatively evaluated by physical examination, chest radiography, and CT of the chest and abdomen to confirm relapse. In some patients, we used PET-CT, magnetic resonance imaging or bone scintigraphy to detect recurrence. The disease stage was determined in accordance with the 7th edition of the TNM classification for lung and pleural tumors.[20]

Histopathology

The available pathology slides from all 532 surgical specimens were reviewed in this study. After fixing the specimens with either 10% formalin and embedding them in paraffin, serial 4-μm sections were stained with hematoxylin and eosin and by elastica van Gieson (EvG) to visualize elastic fibers. Histologic subtypes of lung cancer were determined according to World Health Organization classification.[21] The histological tumor grade was categorized as well-differentiated, moderately differentiated, or poorly differentiated carcinoma according to the degree of structural and cytologic atypia.

Blood vessels were identified by the presence of erythrocytes in the lumen and/or an endothelial cell lining and/or the presence of elastic tissue around larger vessels. Sections stained by EvG were examined for the presence of blood-vessel invasion. The presence of blood-vessel invasion was determined by identifying conspicuous clusters of intravascular cancer surrounded by an elastic layer.

Lymph-vessel invasion was determined to be present when tumor cells floating in lymphatic vessels with no supporting smooth muscles or elastic fibers were identified. We confirmed that lumens within the bronchovascular bundle, subpleural, and intralobular pleural space were lymphatic vessels by immunostaining with anti-D2-40 antibody.

Data Collection

Clinical characteristics were retrieved from available clinical records. The following clinicopathological factors were assessed in the retrospective prognostic analysis: age (dichotomized at the median age of 64 years), sex, smoking status, preoperative serum CEA level (cutoff at the normal upper limit of 5 ng/ml), tumor size, tumor differentiation (well or moderate versus poor), blood-vessel invasion (absence versus presence), lymph-vessel invasion (absence versus presence), histology (adenocarcinoma versus other), tumor laterality, and extent of resection (single-lobe lobectomy versus more extensive resection; bilobectomy or pneumonectomy).

Statistical Analysis

Overall survival (OS) was measured from the date of surgery to the date of death from any cause or the date on which the patient was last known to be alive. The length of the recurrence-free period was calculated in months from the date of resection to the date of the first recurrence or last follow-up showing no recurrence. To calculate the recurrence-free proportion (RFP), patients who died without recurrence or who were known to have no recurrence at the date of last contact were censored. OS and RFP curves were plotted using the Kaplan–Meier method, and differences in variables were determined using the log-rank test. Categorical comparison was performed using the Pearson

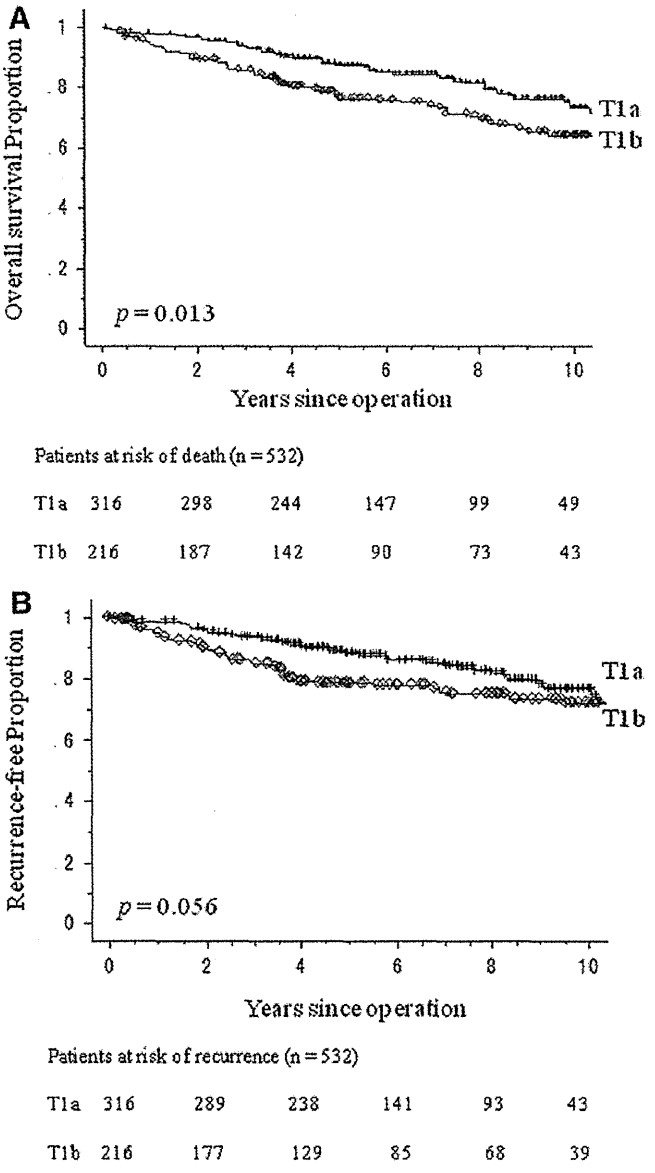

FIGURE 1. *A*, Overall survival curves of patients with T1a or T1b disease. *B*, Recurrence-free proportion curves of patients with T1a or T1b disease.

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χ^2 test. Multivariate analyses were performed using the Cox proportional hazards regression model. All tests were two-sided, and p values of less than 0.05 were considered to indicate a statistically significant difference. Statview 5.0 software (SAS Institute Inc., Cary, NC) was used for statistical analyses. Data collection and analyses were approved and the need to obtain written informed consent from each patient was waived by the institutional review board of our institution.

RESULTS

The median follow-up for survivors was 5.1 years. Figure 1*A* and *B* show the OS and RFP curves of 316 patients with T1aN0M0 NSCLC and 216 patients with T1bN0M0 NSCLC. For those patients with T1aN0M0 NSCLC and those with T1bN0M0 NSCLC, the 5-year OS rates were 87.1% and 77.2% ($p = 0.013$), respectively, whereas the 5-year RFPs were 88.6% and 78.6% ($p = 0.056$), respectively.

Table 1 shows the 5-year OS proportions and RFPs according to the clinicopathological characteristics of the stage IA NSCLC patients. On univariate analysis, nine variables were found to be significantly associated ($p < 0.05$) with poorer OS: older age, male sex, smoking history, T1b, poorly differentiated carcinoma, blood-vessel invasion, lymph-vessel invasion, nonadenocarcinoma, and type of surgery (bilobectomy or pneumonectomy). For RFP, five variables (male sex, poorly differentiated carcinoma, blood-vessel invasion, lymph-vessel invasion, and nonadenocarcinoma) were identified as statistically significant factors on univariate analysis.

A multivariate Cox proportional hazards model demonstrated that older age (hazard ratio [HR] = 1.936; $p < 0.001$), male sex (HR = 2.096; $p = 0.005$), tumor size (HR = 1.501; $p = 0.045$), poorly differentiated carcinoma (HR = 1.632; $p = 0.028$), lymph-vessel invasion (HR = 1.579; $p = 0.042$), and nonadenocarcinoma (HR = 1.704; $p = 0.016$) were statistically significant predictors of OS (Table 2). Poorly differentiated carcinoma (HR = 1.925; $p = 0.006$), blood-vessel invasion (HR = 1.712; $p = 0.020$), and lymph-vessel invasion (HR = 1.751; $p = 0.017$) were identified as statistically significant predictors of cancer recurrence (Table 3). Figures 2*A*, *B*, and *C* show the RFP curves of patients with stage IA NSCLC according to tumor differentiation, blood-vessel invasion, and lymph-vessel invasion, respectively. Table 4 shows the results of 5-year RFP of patients in each T subclassification (T1a and T1b) according to these significant predictors of cancer recurrence.

Subgroup analysis with a combination of these recurrence predictive factors in the patients with stage IA NSCLC revealed 5-year RFPs of 91.3%, 79.5%, and 62.9% for patients with no risk factor, poorly differentiated carcinoma or vascular invasion (blood-vessel invasion or lymph-vessel

TABLE 1. Patient Characteristics and Univariate Analysis of Survival and Recurrence

Variable	No. of Patients	5-Yr OSP (%)	*p* Value	5-Yr RFP (%)	*p* Value
Age (yrs: median 64)					
< 64	279	88.9		84.2	
≥ 64	253	76.6	< 0.001	85.3	0.946
Sex					
Male	290	77.7		81.4	
Female	242	89.6	< 0.001	88.4	0.009
Smoking status					
Ever smoker	279	81.5		82.6	
Never smoker	253	84.9	0.039	86.8	0.102
CEA (ng/ml: NUL of 5)					
< 5	447	83.7		85.2	
≥ 5	59	75.9	0.108	77.2	0.212
Tumor size					
T1a (≤ 2.0 cm)	316	87.1		88.6	
T1b (≥ 2.1 cm)	216	77.2	0.013	78.6	0.056
Differentiation					
Well or moderate	425	86.4		87.7	
Poor	96	71.4	< 0.001	71.8	< 0.001
Blood-vessel invasion					
Absent	402	86.2		88.1	
Present	116	72.1	0.002	71.3	< 0.001
Lymph-vessel invasion					
Absent	392	85.4		87.1	
Present	122	76.4	0.003	76.1	0.001
Histology					
Adenocarcinoma	439	86.6		86.6	
Nonadenocarcinoma	93	66.3	< 0.001	74.3	< 0.001
Tumor laterality					
Right	357	82.9		84.3	
Left	175	83.6	0.685	85.4	0.732
Type of surgery					
Single-lobe lobectomy	510	84.0		84.5	
More extensive resection (more than bilobectomy)	22	66.7	0.046	88.7	0.946

OSP, overall survival proportion; RFP, recurrence-free proportion; NUL, normal upper limit; CEA, preoperative serum carcinoembryonic antigen level.

TABLE 2. Multivariate Cox Proportional Hazards Regression Analysis of Overall Survival

Variable	Risk Factors	Hazard Ratio	95% Confidence Interval	*p* Value
Age	≥ 64	1.936	1.314–2.852	< 0.001
Sex	Male	2.096	1.251–3.510	0.005
Smoking status	Ever smoker	1.219	0.781–1.901	0.383
Tumor size	T1b (≥ 2.1 cm)	1.501	1.009–2.233	0.045
Differentiation	Poor	1.632	1.054–2.527	0.028
Blood-vessel invasion	Present	1.169	0.749–1.827	0.492
Lymph-vessel invasion	Present	1.579	1.017–2.449	0.042
Histology	Nonadenocarcinoma	1.704	1.103–2.632	0.016
Type of surgery	More extensive resection (more than bilobectomy)	1.981	0.984–3.984	0.055

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TABLE 3. Multivariate Cox Proportional Hazards Regression Analysis of Cancer Recurrence

Variable	Risk Factors	Hazard Ratio	95% Confidence Interval	*p* Value
Sex	Male	1.171	0.747–1.834	0.492
Differentiation	Poor	1.925	1.210–3.063	0.006
Blood-vessel invasion	Present	1.712	1.088–2.694	0.020
Lymph-vessel invasion	Present	1.751	1.103–2.779	0.017
Histology	Nonadenocarcinoma	1.615	0.994–2.623	0.053

invasion), and both poorly differentiated carcinoma and vascular invasion, respectively (Fig. 3*A*). The differences in RFP were statistically significant between patients without any risk factors (A group) and those with poorly differentiated carcinoma or vessel invasion (B group) ($p < 0.001$). The 5-year RFP of patients with both poorly differentiated carcinoma and vascular invasion (C group) tended to be unfavorable compared with that of patients in the B group, but the difference was not statistically significant ($p = 0.068$). In patients with T1a, the 5-year RFP of patients without any risk factors (A group) was statistically different from that of patients with poorly differentiated carcinoma or vessel invasion (B group) (92.0% versus 83.7% in A and B, respectively; $p = 0.002$), whereas

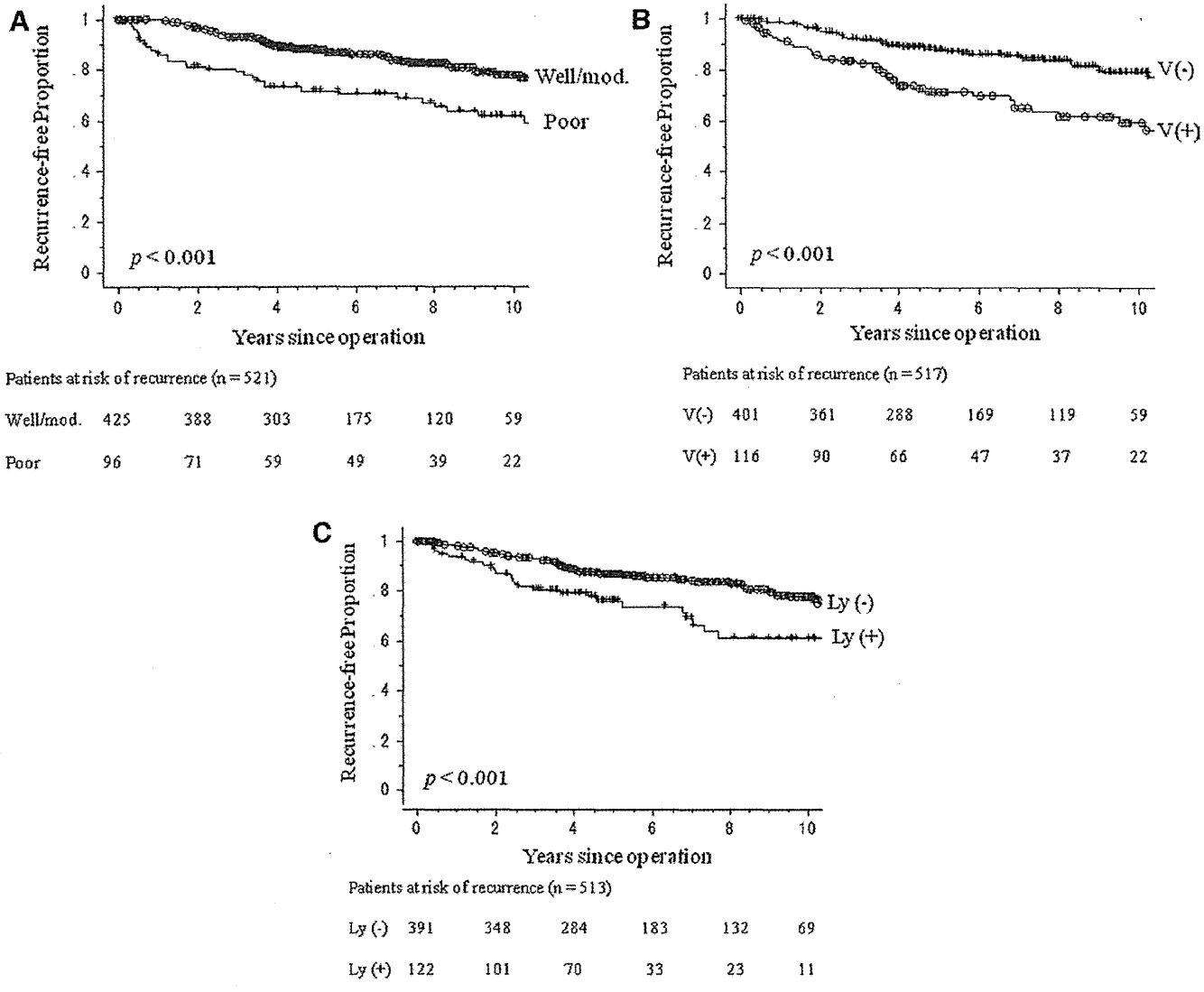

FIGURE 2. *A*, Recurrence-free proportion curves according to tumor differentiation. *B*, Recurrence-free proportion curves according to blood-vessel invasion. *C*, Recurrence-free proportion curves according to lymph-vessel invasion.

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TABLE 4. 5-Year Recurrence-Free Proportion for Each T Subclassification According to Histological Grade and Vascular-Invasion Status

T-Factor category	No. of Patients	5-Yr RFP (%)	*p* Value
T1a (≤ 2.0 cm)			
Well/mod.	249	90.3	
Poor	60	83.8	0.126
T1b (≥ 2.1 cm)			
Well/mod.	176	83.7	
Poor	36	51.3	< 0.001
T1a (≤ 2.0 cm)			
BVI (−)	265	90.2	
BVI (+)	44	77.5	0.005
T1b (≥ 2.1 cm)			
BVI (−)	137	83.8	
BVI (+)	72	67.0	0.011
T1a (≤ 2.0 cm)			
LVI (−)	252	90.2	
LVI (+)	54	79.4	0.003
T1b (≥ 2.1 cm)			
LVI(−)	140	81.4	
LVI (+)	68	73.2	0.181

RFP, recurrence-free proportion; Well/mod., well- or moderately differentiated carcinoma; Poor, poorly differentiated carcinoma; BVI, blood-vessel invasion; LVI, lymph-vessel invasion.

no significant difference was shown between patients in the B group and those with both poorly differentiated carcinoma and vascular invasion (C group; 79.4% at 5-year RFP for C group; $p = 0.812$) (Fig. 3*B*). The RFP curves for T1b patients of the A, B, and C groups were shown in Fig. 3*C*. The differences in recurrence were statistically significant between A and B (89.6% versus 75.1% at 5-year RFP in A and B, respectively; $p = 0.006$), B and C (43.3% at 5-year RFP for the C group; $p = 0.002$).

We tested for a correlation between histological grade or vascular-invasion status and clinicopathological variables in stage IA patients. A comparison of variables between well- or moderately differentiated carcinoma and poorly differentiated carcinoma groups showed that a statistically significant difference in the prevalence of poorly differentiated carcinoma was seen in patients of male sex ($p < 0.001$), those who were smokers ($p < 0.001$) those in whom vascular invasion was present ($p < 0.001$), and those who had nonadenocarcinoma histology ($p < 0.001$). Vascular invasion was significantly associated with male sex ($p = 0.035$), smoking ($p = 0.001$), T1b ($p < 0.001$), and poorly differentiated carcinoma ($p < 0.001$) (data not shown).

Table 5 shows the number of patients with recurrence and their initial recurrence pattern according to histological grade and vascular-invasion status. The proportion of patients who developed distant metastases was higher in these recurrence predictive factor positive populations than in the negative populations (histological grade; $p = 0.048$, vascular invasion; $p = 0.024$).

DISCUSSION

We set out to identify the clinicopathological factors that affect overall prognosis and cancer recurrence of stage IA NSCLC. Curative surgical resection is the most effective therapy for patients with stage IA NSCLC. However, a considerable number of patients develop recurrence, which results in cancer death. Previous studies have reported the following factors to be associated with a poor prognosis in patients with stage IA NSCLC: tumor size,[5] preoperative serum CEA level,[7] lymph-vessel invasion,[18] blood-vessel invasion,[7,13–15,17] and histological grade.[14,17,19] In addition, according to the Surveillance, Epidemiology, and End Result Program database, age, sex, and extent of resection are also important prognostic factors.[22] However, prognostic factors such as age and sex do not accurately predict or explain recurrence in patients with stage IA NSCLC. Therefore, we focused on the risk factors for recurrence and unfavorable OS in the present study. When describing the survival experience of a group of patients, the OS parameter is typically used. However, OS is affected by death resulting from causes other than lung cancer itself, including complications and comorbidities, and is considered to be affected by treatment after relapse. For example, epidermal growth factor receptor tyrosine kinase inhibitors are highly effective against mutated epidermal growth factor receptor recurrent NSCLC patients, suggesting potential improvements in postoperative survival regardless of surgery effect. Therefore, in evaluating pure surgical impact on the natural history of early-stage NSCLC, we consider that RFP may be a better prognostic indicator than OS. On multivariate analyses, we identified five independently significant predictors for poor prognosis: older age (HR = 1.936), male sex (HR = 2.096), tumor size (HR = 1.501), poorly differentiated carcinoma (HR = 1.632), lymph-vessel invasion (HR = 1.579), and nonadenocarcinoma (HR = 1.704); we also identified three predictors of recurrence: poorly differentiated carcinoma (HR = 1.925), blood-vessel invasion (HR = 1.712), and lymph-vessel invasion (HR = 1.751). The present study showed that independent predictive factors of poor survival were slightly different from predictive factors of recurrence.

Several authors reported that patients with poor differentiated carcinomas after resection had a higher risk of recurrence and death.[14,23,24] Although the histological grading system may provide useful information in defining the aggressiveness of tumors and has a significant impact on the survival of patients,[19] the four-tiered system of grading (well-differentiated, moderately differentiated, poorly differentiated, and undifferentiated carcinomas) for lung cancer is assumed to lack objectivity, because no original criteria have been developed for standardizing lung cancer histology. However, the current result indicates that poor differentiation contributes to unfavorable clinical outcome, suggesting that this factor may be a useful indicator of a need for postoperative adjuvant chemotherapy in patients with stage IA NSCLC. Consistent grading criteria need to be established for reproducible assessment.

Blood-vessel invasion is considered to be a fundamental step in hematogenous metastasis. The presence of blood-vessel invasion was previously found to be a strong

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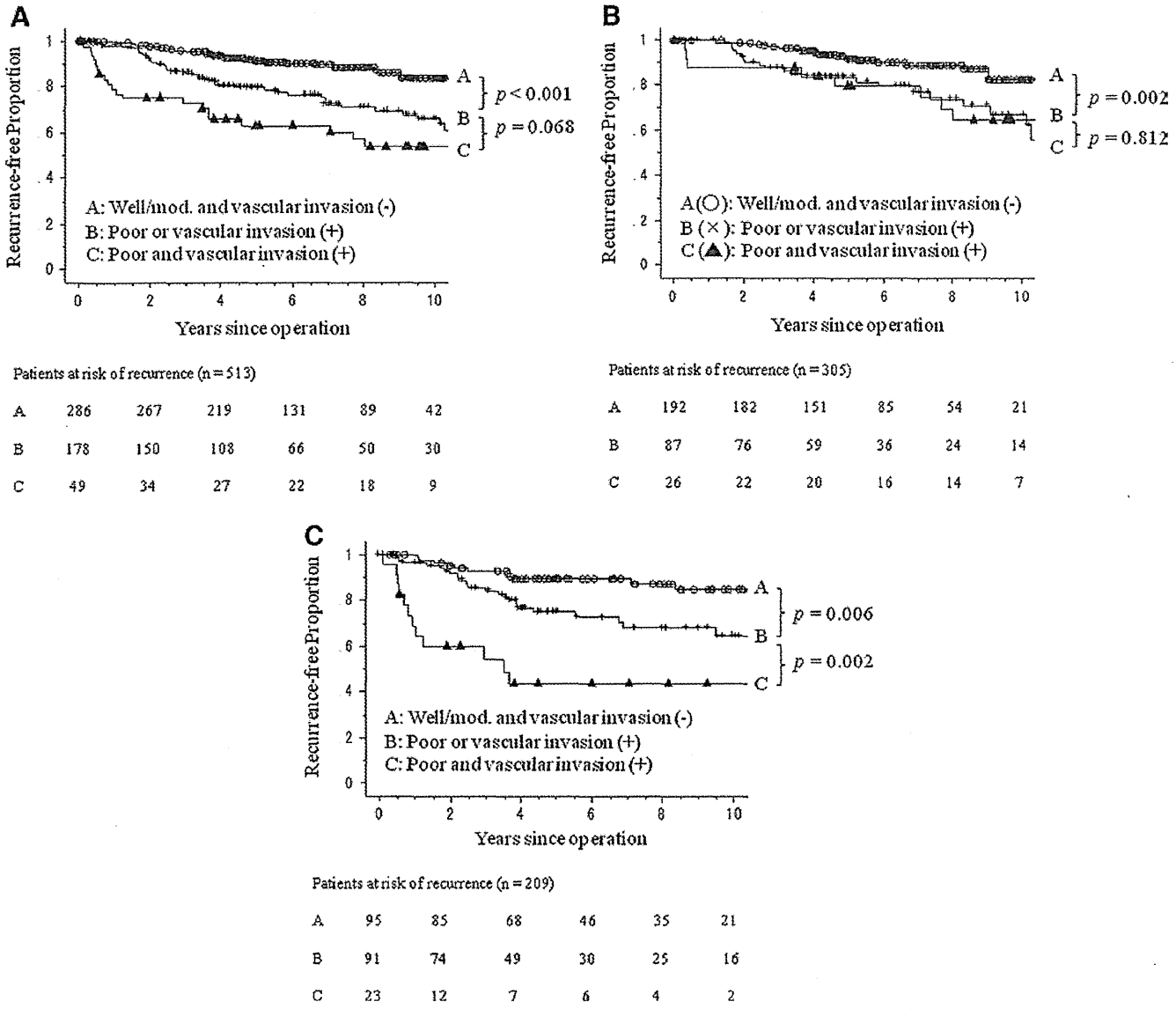

FIGURE 3. *A*, Recurrence-free proportion curves for all stage IA; *B*, T1a; and *C*, T1b patients with well- or moderately differentiated carcinoma and no vascular invasion (curve A), poorly differentiated carcinoma or vascular invasion (curve B), and both poorly differentiated carcinoma and vascular-invasion (curve C).

independent unfavorable prognostic factor, and vascular invasion should be considered for inclusion in the staging criteria and indications for adjuvant chemotherapy.[10,11,13] Fujisawa et al.[25] demonstrated that blood-vessel invasion is a very important prognostic factor in resected NSCLCs with intrapulmonary metastasis, and may correlate with the anatomical aspect of pulmonary metastasis. The current study also suggests that the presence of blood-vessel invasion is a significant risk factor for recurrence in stage IA NSCLC patients.

To identify blood-vessel invasion more accurately, we used hematoxylin and eosin and EvG stains to visualize elastic fibers in all cases. We recommend the routine use of elastic stains in the pathological evaluation of lung cancer, not only for the determination of visceral pleural invasion but also for the determination of blood-vessel invasion, particularly in patients with stage IA NSCLC.

Lymph-vessel invasion has been reported to be an independent indicator of cancer invasiveness and poor prognosis in most studies that included this factor in their analyses.[9,18,26,27] The present study shows that as it is for histological grade, lymph-vessel invasion was a significant predictor of both poor prognosis and cancer recurrence, surpassing tumor size in pathologic stage IA NSCLC.

Recent randomized controlled trials have demonstrated the usefulness of postoperative adjuvant chemotherapy in stage IB to IIIA NSCLC patients who have undergone complete resections.[28–30] Although surgery alone remains the

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TABLE 5. Initial Observed Cancer Recurrence Patterns of Patients According to Histological Grade and Vascular-Invasion Status

Initial Recurrence Pattern	Tumor Differentiation			Vascular Invasion		
	Well/ mod.	Poor	*p* Value	Absent	Present	*p* Value
Overall (%)	425 (82)	96 (18)		340 (65)	180 (35)	
Patients with recurrence (%)	64 (15)	35 (36)		46 (14)	53 (29)	
Local recurrence only	24 (38)	9 (25)	0.048	19 (41)	14 (26)	0.025
Distant recurrence	39 (62)	27 (75)		27 (59)	39 (74)	

Well/mod., well- or moderately differentiated carcinoma; Poor, poorly differentiated carcinoma.

standard treatment for patients with stage IA NSCLC, larger studies on resected cases comparing uracil-tegafur adjuvant chemotherapy versus observation showed that uracil-tegafu-rimproved survival for patients with stage I adenocarcinoma, and also showed a clear survival benefit in the T1-disease subgroup of patients with a tumor of diameter more than 2 cm.[31,32] However, tumor size might not be the only factor found to have a benefit on adjuvant chemotherapy after complete resection of stage IA NSCLC. In the present study, when we divided the study population into A (patients without any risk factors), B (those with either poorly differentiated carcinoma or vascular invasion), and C (those with both poorly differentiated carcinoma and vascular invasion) groups, the 5-year RFP of all stage IA patients were 91.3%, 79.5%, and 62.9%, respectively. In particular, the subgroup analysis of patients with stage IA disease stratified by tumor size showed a 5-year RFP of 43.3% for the T1b C group. These results indicated high-risk small-tumor N0 patiénts, identified by factors other than tumor size, such as tumor differentiation and vascular invasion, may be good candidates for adjuvant chemotherapy.

This study has limitations and biases that should be mentioned. As a retrospective single-institute study, patient-selection bias and time-trend bias regarding the diagnosis for cancer recurrence might be inevitable compared with multi-institutional prospective study. Moreover, the definition of an ipsilateral lung metastasis as a local recurrence also generated inherent bias while allowing the differentiation of a new primary lung cancer from a recurrent NSCLC.

The anatomical extent of disease, as described by the TNM for lung and pleural tumors, remains the most powerful prognostic instrument in NSCLC. A challenge for the future will be to integrate the TNM with specific pathological factors, such as vascular-invasion status or tumor differentiation, to create a composite prognostic index for NSCLC.

CONCLUSION

Even though most patients comprised an early-staging subset, those with stage IA NSCLC comprised a heterogeneous group with different prognoses and risk of cancer recurrence. The current study demonstrates that vascular-invasion status and tumor differentiation were far more powerful recurrence predictive factors than tumor size, and this information can be useful for the selection of the appropriate therapeutic strategy, including adjuvant chemotherapy, which can be tailored to the individual patient's risk of developing recurrence.

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Impact of visceral pleural invasion on the survival of patients with non-small cell lung cancer

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ABSTRACT

Background: In this study, we investigated visceral pleural invasion (VPI) as a poor prognostic factor in patients with non-small cell lung cancer (NSCLC) according to the 7th edition of the TNM classification.
Methods: Between January 2000 and December 2007, 886 consecutive patients with pathological T1a–T2b NSCLC underwent complete resection with systematic lymph node dissection in Tokyo Medical University. We statistically analyzed the association between VPI and clinicopathologic factors, or clinical outcomes.
Results: The 5-year overall survival (OS) rates of the pl0, pl1, and pl2 patients were 80.8%, 63.7%, and 49.6%, respectively, with significant differences between pl0 and pl1 ($p=0.002$), pl1 and pl2 ($p=0.03$). Thus, the pl1 and pl2 patient groups were defined as patients with VPI. VPI was found to be a significant independent prognostic factor by multivariate survival analysis ($p=0.0002$). In patients with tumors ≤3 cm, especially with tumors ≤2 cm, VPI was significantly associated with an increased rate of lymph node metastasis, compared with non-VPI ($p=0.0003$ and $p=0.015$, respectively). Analysis of the OS of patients stratified by tumor size (≤3 cm, 3.1–5 cm, 5.1–7 cm) and VPI status showed that in any nodal status, patients with 3.1–5 cm/VPI tumors had significantly worse survival than patients with ≤3 cm/VPI tumors ($p=0.019$) and patients with 3.1–5 cm/non-VPI tumors ($p=0.001$). On the other hand, there was no significant difference in the OS between patients with 3.1–5 cm/VPI tumors and patients with 5.1–7 cm tumors regardless of lymph node metastasis (T2b tumors). Similar relationships were observed among these groups with N0 disease.
Conclusion: We identified the presence of VPI as an independent poor prognostic factor in patients with NSCLC of ≤7 cm. Tumors 3.1–5 cm with VPI should be upstaged to T2b tumors in the future in the TNM classification of the Union of International Cancer Control staging system. In addition, the surgical strategy involving more extensive lymph node dissection for patients with ≤3 cm/VPI tumors, especially ≤2 cm/VPI, is warranted owing to more frequent lymph node metastasis.
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1. Introduction

The report on the monitoring of cancer incidence in Japan estimates that there were approximately 85,500 patients with lung cancer in 2006. Complete resection, whenever feasible, is generally recognized as the most effective initial treatment for NSCLC. However, even with complete resection, the 5-year survival rates are disappointing and range from 79.4–83.9% for stage IA, to 29.8–32.8% for stage IIIA [1–3].

The TNM stage is the most important prognostic factor in NSCLC. The prognostic factors in patients who have undergone resection may enable identification of groups with poor survival who could benefit from combination therapy (adjuvant chemotherapy or radiotherapy). Visceral pleural invasion (VPI) of lung cancer has been reported as a poor prognostic factor [4–10].

In the present study, we retrospectively investigated the prognostic impact of VPI in the context of other established clinical prognostic factors, in a series of 886 consecutive NSCLC patients who underwent complete resection at our hospital. Furthermore, we analyzed the effects of combination of VPI and tumor size for

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T-size categories, particularly T2a tumors, using the 7th edition of the TNM classification.

2. Materials and methods

2.1. Patients

From January 2000 to December 2007, a total of 1145 patients underwent complete resection of NSCLC at our hospital. The purpose of this study was to determine whether tumor invasion beyond the elastic layer of the pleura or the pleural surface is associated with poor prognosis, lymph node metastasis, or other clinicopathologic factors. Thus, the pl1 and pl2 patient groups were defined as having VPI and we excluded pl3 patients. As T3 tumors invaded the parietal pleura (pl3), have pulmonary metastasis in the same lobe, or were >7 cm, even if we exclude only patients with pl3 tumors from this study, there were still patients whose T3 tumors had other prognostic factors. Thus, we excluded patients with T3 tumors and those who had received preoperative chemotherapy, radiotherapy or both, or who had been given a diagnosis of low-grade malignant histologies, including carcinoid, mucoepidermoid carcinoma, and adenoid cystic carcinoma. The remaining 886 patients who were pathologically confirmed to have T1a–T2b NSCLC were enrolled in this study. We defined complete resection as lobectomy or more extensive lung resection with systematic ipsilateral hilar and mediastinal lymph node dissection and with no evidence of residual cancer either macroscopically or microscopically. We reviewed the medical records of each patient regarding their clinicopathologic information including age, gender, pathological nodal involvement, pleural invasion, tumor location, and histologic type. Disease stages were based on the 7th edition of the TNM Classification for Lung and Pleural Tumors of the Union for International Cancer Control (UICC). The median follow-up period was 4.6 years. After resection, the patients were examined at 3-month intervals for 3 years, at 6-month intervals for the next 2 years, and thereafter at 1-year intervals in general. The evaluations included physical examination, chest roentgenogram, chest CT, and tumor marker measurement. Abdominal and brain CT as well as bone scintigraphy were performed each year. Patients with cancer recurrences were carefully divided into two groups according to the site of initial relapse: locoregional or distant. Locoregional recurrence was defined as any recurrent site within the ipsilateral hemithorax, mediastinum, or supraclavicular lymph nodes. All other sites of recurrence were considered distant metastases. The Institutional Review Board of our hospital approved the protocols for data collection and analyses, and waived the need to obtain written informed consent from each patient.

2.2. Histopathologic studies

Histopathologic studies were performed according to World Health Organization criteria. Detailed examinations of VPI were routinely performed at our institution. Tumor sections were stained with hematoxylin and eosin and elastica van Gieson (EVG), which allows the pathologist to assess the integrity of the pleural elastic layer and makes it easier to identify pleural invasion by the tumor and to determine the pathologic stage of the disease. VPI was classified according to the 7th edition of the TNM Classification for Lung and Pleural Tumors as follows: pl0 is defined as a tumor within the subpleural lung parenchyma or which invades superficially into the pleural connective tissue beneath the elastic layer; pl1 is defined as a tumor that invades beyond the elastic layer; pl2 is defined as a tumor that invades the pleural surface; and pl3 is defined as a tumor that invades any component of the parietal pleura (Fig. 1).

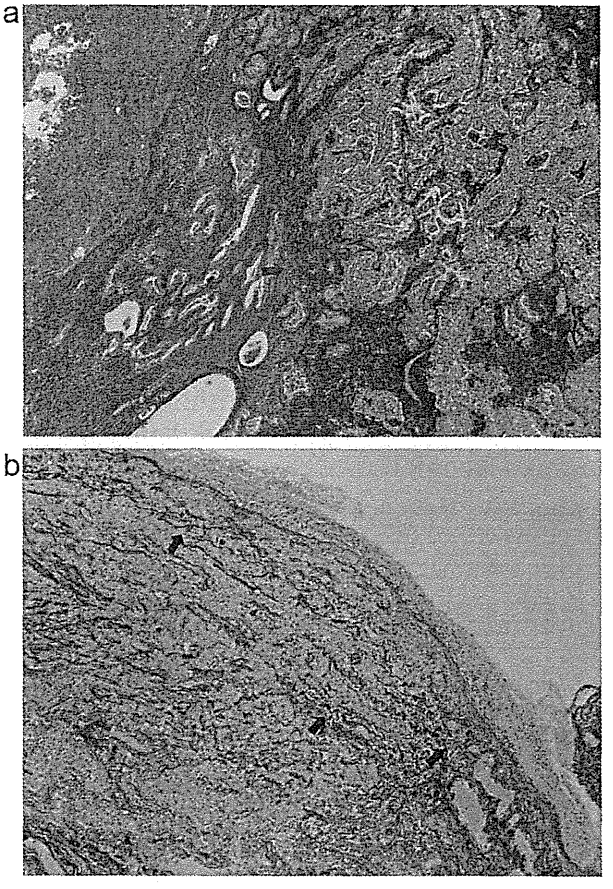

Fig. 1. Elastica van Gieson staining of a pl1 status tumor. The tumor cells extend beyond the elastic layer (arrow) of the visceral pleura, but are not exposed on the pleural surface (original magnification, 40×). Elastica van Gieson staining of a pl2 status tumor. The tumor cells extend beyond the elastic layer (arrow) of the visceral pleura, and are also exposed on the pleural surface (original magnification, 100×).

2.3. Statistical analysis

Overall survival (OS) and recurrence-free survival (RFS) were estimated using the Kaplan–Meier method, and differences in survival rates were determined by log-rank analysis. OS was defined as the time elapsed from the date of pulmonary resection to the date of death. RFS was defined as the time elapsed from the date of pulmonary resection to the date of the first recurrence or last follow-up showing no recurrence. The last follow-up observation was censored if the patient was alive or lost to follow-up. Univariate analysis was conducted among the different groups. Categorical variables were analyzed using the chi-square test. Differences between 2 groups were tested using the Student *t*-test. Multivariate analysis was performed by constructing the Cox proportional hazards model using the significant factors identified from univariate analysis to examine the association between survival and potential prognostic factors. All *p*-values were two-sided and p-values < 0.05 were considered to indicate a statistically significant difference. All statistical calculations were performed using StatView for Windows version 5.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

The characteristics of the patients are shown in Table 1a. The degrees of VPI were pl0 in 692 patients (78.1%), pl1 in 132 (14.9%), and pl2 in 62 (7.0%). The OS curves of the degree of VPI are shown

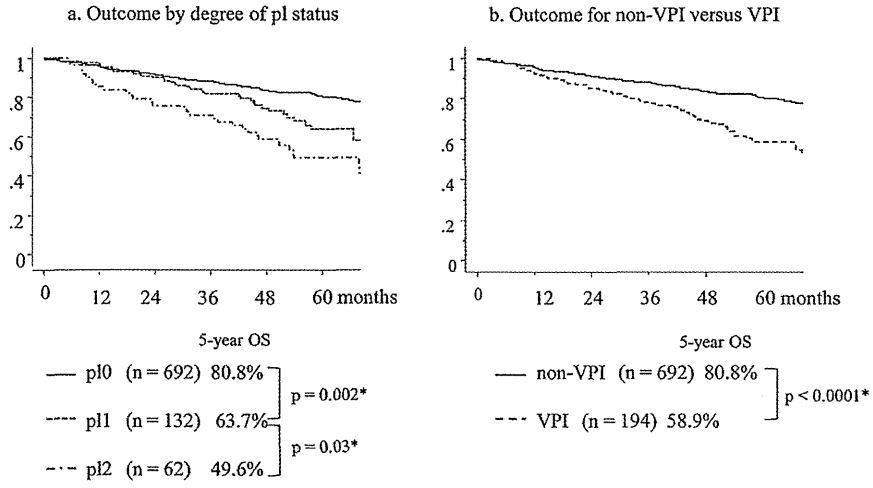

Fig. 2. Survival curves based on the degree of visceral pleural invasion (VPI) in all patients. The differences in survival between pl0 tumors and pl1 tumors ($p = 0.002$), and between pl1 tumors and pl2 tumors ($p = 0.03$) were statistically significant. Survival curves for non-VPI and VPI. There were significant differences in survival between non-VPI and VPI ($p < 0.0001$).

in Fig. 2a. The 5-year OS rates of the pl0, pl1, and pl2 patients were 80.8%, 63.7%, and 49.6%, respectively. The differences in survival rates were statistically significant between pl0 and pl1 ($p = 0.002$), pl1 and pl2 ($p = 0.03$). The OS curves of the patients according to the presence or absence of VPI, including pl1 and pl2, are shown in Fig. 2b. The 5-year OS rates of non-VPI and VPI patients were 80.8% and 58.9%, respectively, and the difference was statistically significant ($p < 0.0001$).

Table 1a
Patient characteristics ($n = 886$).

Variable	Category	Number (%)
Sex	Men	508 (57.3)
	Women	378 (42.7)
Median age (range)		66.0 (22–87)
Smoking habits	Smoker	526 (57.8)
	Non-smoker	350 (42.7)
Tumor location	Right	568 (64.1)
	Left	318 (35.9)
Histological type	Adenocarcinoma	675 (76.2)
	Squamous cell carcinoma	157 (17.7)
	Large cell carcinoma	40 (4.5)
	Others	14 (1.6)
Operation procedure	Lobectomy/bilobectomy	878 (99.1)
	Pneumonectomy	8 (0.9)
Mean tumor size (cm) (range)		2.7 (0.4–7.0)
pT factor	T1a	276 (31.2)
	T1b	190 (21.4)
	T2a	372 (42.0)
	T2b	48 (5.4)
pN factor	N0	708 (79.9)
	N1	100 (11.3)
	N2	78 (8.8)
p-Stage	IA	415 (46.8)
	IB	264 (29.8)
	IIA	116 (13.1)
	IIB	13 (1.5)
	IIIA	78 (8.8)
Visceral pleural invasion	pl0	692 (78.1)
	pl1	132 (14.9)
	pl2	62 (7.0)

The relationship between clinicopathologic prognostic factors and VPI is shown Table 1b. There were significantly more tumors with VPI in patients who are men, are smokers, have non-adenocarcinoma, and are positive for lymph node metastasis ($p < 0.05$). Tumor size was significantly larger in VPI tumors than in non-VPI tumors (median: 3.0 cm and 2.5 cm, respectively; $p < 0.0001$). There was no significant difference between VPI tumors and non-VPI tumors with regard to several clinical factors including age, tumor location, and operation procedure. The association between VPI and lymph node metastasis are summarized in Fig. 3. In patients with tumors ≤3 cm, VPI was more significantly associated with an increased rate of lymph node metastasis, compared with non-VPI ($p = 0.0003$). Furthermore, especially in tumors ≤2 cm, VPI tumors had lymph node metastasis in 8 patients (20.0%), and non-VPI had in 22 (7.9%), with significant differences ($p = 0.015$, data not shown). On the other hand, in tumors >3 cm, there was no significant association between VPI and lymph node metastasis (3.1–5 cm tumors, $p = 0.20$; 5.1–7 cm tumors, $p = 0.86$).

The potential prognostic factors were analyzed using univariate survival analysis (Table 2), which showed that sex, age, smoking habits, tumor size, histologic type, lymph node metastasis, and VPI were significant prognostic factors. Multivariate survival analysis (Table 3) showed that VPI, sex, age, tumor size, and lymph node metastasis were significant independent prognostic factors (relative risk = 1.75, $p = 0.0002$).

We analyzed the OS of patients stratified by tumor size (≤3 cm, 3.1–5 cm, 5.1–7 cm) and VPI status (presence or absence) (Fig. 4a). Subgroup analysis of the patients, regardless of lymph node metastasis, revealed 5-year OS rates of 85.8%, 70.1%, 73.2%, 53.5%, 51.4%, and 32.1% for patients with ≤3 cm/non-VPI, ≤3 cm/VPI, 3.1–5 cm/non-VPI, 3.1–5 cm/VPI, 5.1–7 cm/non-VPI, and 5.1–7 cm/VPI tumors, respectively. The difference in the OS rate was statistically significant between the ≤3 cm/non-VPI and ≤3 cm/VPI subgroups ($p = 0.010$; Fig. 4a-i), and the 3.1–5 cm/non-VPI and 3.1–5 cm/VPI subgroups ($p < 0.001$; Fig. 4a-ii). In contrast, there was no significant difference in the survival rates between the 5.1–7 cm/non-VPI and 5.1–7 cm/VPI subgroups ($p = 0.68$; Fig. 4a-iii).

In the pN0 patient cohort, subgroup analysis of the patients revealed 5-year OS rates of 86.6%, 75.9%, 79.3%, 66.4%, 59.7%, and 46.2% for patients with ≤3 cm/non-VPI, ≤3 cm/VPI, 3.1–5 cm/non-VPI, 3.1–5 cm/VPI, 5.1–7 cm/non-VPI, and 5.1–7 cm/VPI tumors, respectively. The 5-year OS rate of patients with ≤3 cm/non-VPI tumors was worse than that of patients with ≤3 cm/VPI, although

Table 1b
Patient characteristics in the 2 groups according to clinicopathologic factors ($n = 886$).

Variable	Number (%)		p-Value [non-VPI vs. VPI]
	Non-VPI ($n = 692$)	VPI ($n = 194$)	
Sex			
Men	378 (54.6)	130 (67.0)	0.02*
Women	314 (45.4)	64 (33.0)	
Median age (range)	66.0 (22–85)	66.0 (27–87)	0.92
Smoking habits			
Smoker	398 (57.5)	128 (66.0)	0.03
Non-smoker/unknown	294 (42.5)	66 (34.0)	
Tumor location			
Right	448 (64.7)	120 (61.9)	0.46
Left	244 (35.3)	74 (38.1)	
Histological type			
Adenocarcinoma	538 (77.7)	137 (70.6)	0.04
Squamous cell carcinoma	119 (17.2)	39 (20.1)	(Ad
Large cell carcinoma	28 (4.1)	13 (6.7)	vs.
Others	7 (1.0)	5 (2.6)	non-
Operation procedure			Ad)
Lobectomy/bilobectomy	688 (99.4)	190 (97.9)	0.054
Pneumonectomy	4 (0.6)	4 (2.1)	
Median tumor size (cm) (range)	2.5 (0.4–7.0)	3.0 (0.6–7.0)	
pT factor			
T1a	276 (39.9)	–	<0.0001*
T1b	190 (27.4)	–	
T2a	200 (28.9)	172 (88.7)	
T2b	26 (3.8)	22 (11.3)	
pN factor			
N0	574 (82.9)	134 (69.0%)	<0.0001*
N1	75 (10.9)	25 (12.9)	
N2	43 (6.2)	35 (18.1)	
pStage			
IA	415 (60.0)	–	<0.0001*
IB	143 (20.7)	121 (62.4)	
IIA	83 (12.0)	33 (17.0)	
IIB	8 (1.1)	5 (2.6)	
IIIA	43 (6.2)	35 (18.0)	

Non-VPI, without visceral pleural invasion; VPI, with visceral pleural invasion. Ad, adenocarcinoma; Non-Ad, other histological types.
* $p < 0.05$.

not statistically significantly ($p = 0.12$; Fig. 4a-iv). The difference was statistically significant between the 3.1–5 cm/non-VPI and 3.1–5 cm/VPI subgroups ($p = 0.040$; Fig. 4a-v). There was no significant difference in the survival rate between the 5.1–7 cm/non-VPI and 5.1–7 cm/VPI subgroups ($p = 0.980$; Fig. 4a-vi).

We also analyzed the prognosis of T2a and T2b tumors (Table 3a). In the patients with or without lymph node metastasis, the patients with 3.1–5 cm/VPI tumors had significantly worse survival than the patients with ≤3 cm/VPI ($p = 0.002$). There was no significant difference in survival between the patients with 3.1–5 cm/VPI tumors and the patients with 5.1–7 cm/non-VPI, and between the patients with 3.1–5 cm/VPI tumors and the patients with 5.1–7 cm/VPI ($p = 0.87$ and $p = 0.53$, respectively). Although the difference in survival was not statistically significant between the

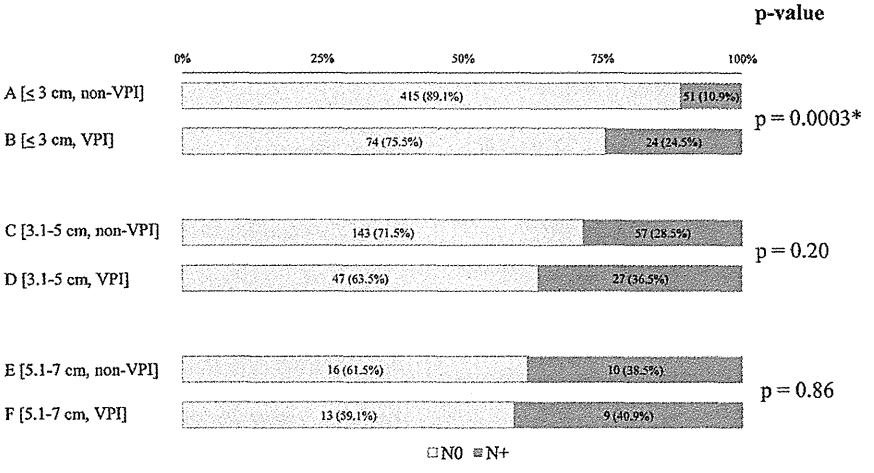

Fig. 3. Relationship between lymph node metastasis and VPI for each tumor size. Only ≤3 cm/VPI tumors significantly developed lymph node metastasis more frequently ($p = 0.0003$). No significant differences in the frequency of lymph node metastasis between tumors with VPI and tumors without VPI were observed in tumors >3 cm (3.1–5 cm, $p = 0.20$; 5.1–7 cm, $p = 0.86$, respectively). N0, no regional lymph node metastasis; N+, with regional lymph node metastasis.

Table 2
Univariate and multivariate analyses of prognostic factors.

Variable	UVA	MVA		
	p-Value	Hazard ratio	95% CI	*p*-Value
Sex: men (vs. women)	<0.0001*	1.35	0.93–2.00	0.11
Age: >70 (vs. ≤70)	0.02*	1.33	1.01–1.79	0.04*
Smoking habits: smoker (vs. non-smoker)	<0.0001*	1.18	0.80–1.79	0.41
Operation procedure: pneumonectomy (vs. others)	NS (0.11)		Not included in MVA	
Tumor location	NS (0.16)		Not included in MVA	
Tumor size: >3 cm (vs. ≤3 cm)	<0.0001*	1.69	1.27–2.22	0.003*
Histologic type: non-Ad (vs. Ad)	<0.0001*	1.31	0.96–1.78	0.09
Lymph node metastasis: present (vs. absent)	<0.0001*	2.38	1.78–3.23	<0.0001*
Visceral pleural invasion: present (vs. absent)	<0.0001*	1.75	1.32–2.38	0.0002*

UVA, univariate analysis; MVA, multivariate analysis. Ad, adenocarcinoma; Non-Ad, other histological types.
* $p < 0.05$.

patients with ≤3 cm/VPI tumors and the patients with 3.1–5 cm/VPI ($p = 0.07$), similar relationships were observed among these groups with N0 disease.

The RFS curves are shown in Fig. 4b. The difference in the RFS rate was statistically significant between the non-VPI and VPI subgroups in tumors ≤5 cm, regardless of lymph node metastasis.

4. Discussion

We set out to investigate the impact of VPI on the survival of NSCLC patients. Pleural invasion by lung cancer was recognized as early as 1958 by Brewer et al. to be a poor prognostic factor for lung cancer [11]. VPI of lung cancer has been shown by several

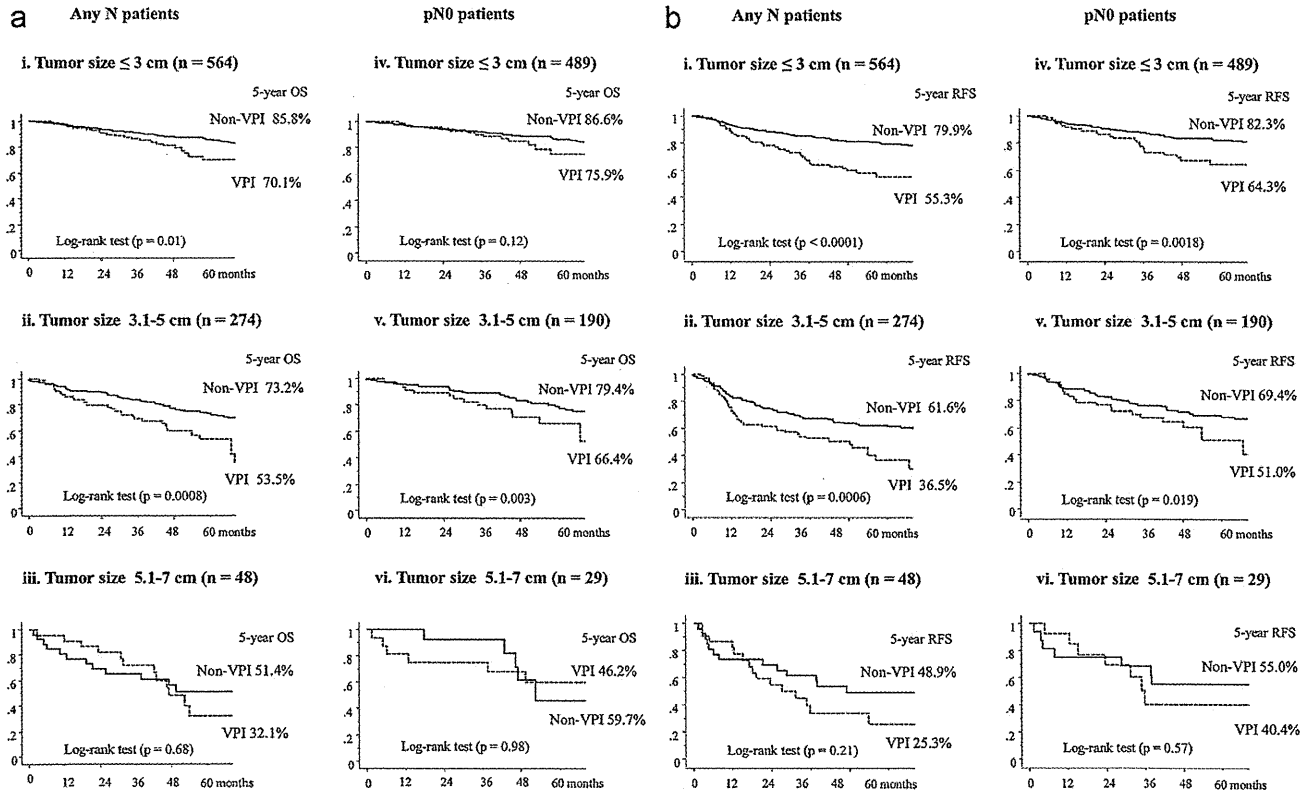

Fig. 4. (a) Survival curves and 5-year overall survival rates stratified by tumor size (≤3 cm, 3.1–5 cm, 5.1–7 cm) and VPI status (presence or absence). (i) Survival curves for the presence or absence of VPI in tumors ≤3 cm, regardless of lymph node metastasis. (ii) Survival curves for the presence or absence of VPI in 3.1–5 cm tumors, regardless of lymph node metastasis. (iii) Survival curves for the presence or absence of VPI in 5.1–7 cm tumors, regardless of lymph node metastasis. (iv) Survival curves for the presence or absence of VPI in tumors ≤3 cm without lymph node metastasis. (v) Survival curves for the presence or absence of VPI in 3.1–5 cm tumors without lymph node metastasis. (vi) Survival curves for the presence or absence of VPI in 5.1–7 cm tumors without lymph node metastasis. Any N patients, patients regardless of lymph node metastasis; pN0 patients, patients with lymph node metastasis; 5-y OS, 5-year overall survival rate. (b) Recurrence-free survival curves and 5-year recurrence-free survival rates stratified by tumor size (≤3 cm, 3.1–5 cm, 5.1–7 cm) and VPI status (presence or absence). (i) Recurrence-free survival curves for the presence or absence of VPI in tumors ≤3 cm, regardless of lymph node metastasis. (ii) Recurrence-free survival curves for the presence or absence of VPI in 3.1–5 cm tumors, regardless of lymph node metastasis. (iii) Recurrence-free survival curves for the presence or absence of VPI in 5.1–7 cm tumors, regardless of lymph node metastasis. (iv) Recurrence-free survival l curves for the presence or absence of VPI in tumors ≤3 cm without lymph node metastasis. (v) Recurrence-free survival curves for the presence or absence of VPI in 3.1–5 cm tumors without lymph node metastasis. (vi) Recurrence-free survival curves for the presence or absence of VPI in 5.1–7 cm tumors without lymph node metastasis. Any N patients, patients regardless of lymph node metastasis; pN0 patients, patients with lymph node metastasis; 5-y RFS, 5-year recurrence-free survival rate.

Table 3a
Overall survival of patients stratified by tumor size and VPI in the entire cohort and pN0 cohort.

	Group	Any pN				pN0			
		n	5-y OS	p-value		n	5-y OS	p-value	
A	≤3 cm, non-VPI	466	85.8	*0.01		415	86.6	0.12	
B	≤3 cm, VPI	98	70.1	0.69	*0.02	74	75.9	0.96	0.07
C	3.1-5 cm, non-VPI	200	73.2	*0.0008		143	79.4	*0.003	
D	3.1-5 cm, VPI	74	53.5	0.87	0.53	47	66.4	0.94	0.77
E	5.1-7 cm, non-VPI	26	51.4	0.68		16	59.7	0.98	
F	5.1-7 cm, VPI	22	32.1			13	46.2		

Non-VPI, without visceral pleural invasion; VPI, with visceral pleural invasion. Any pN, patients regardless of lymph node metastasis (the entire cohort); pN0, patients without lymph node metastasis; 5-y OS, 5-year overall survival rate.
*$p < 0.05$.

studies as a poor prognostic factor [4–10], although other reports have found otherwise [12,13]. VPI appeared in the mid-1970s as a specific entity in the TNM classification. This assigned a tumor of any size that invades the visceral pleura as T2 in the 1997 "Revisions in the International System for Staging Lung Cancer" by Mountain [14]. This has remained unchanged until the 6th edition.

In the 7th edition of the TNM Classification for Lung and Pleural Tumors, VPI is defined as comprising both pl1 and pl2 [15,16]. Despite concerns regarding the effects of the degree of VPI on the T status, the TNM staging system states that only tumors ≤3 cm (T1a and T1b) with VPI are upgraded as T2a, whereas tumors >3 cm and ≤7 cm (T2a and T2b) with VPI remain as T2. However, several reports have shown that patients with VPI whose tumors were >3 cm may be considered to be upgraded [7,10,17,18]. In the present series, we analyzed the impact of VPI on NSCLC patient survival and propose a method of incorporating VPI into the T status, in relation to the 7th TNM classification. The OS curves of pl0, pl1, and pl2 patients are shown in Fig. 2. Cox hazard regression model analysis showed that the division of "pl0 vs. pl1 and pl2" (HR = 0.57, 95% CI: 0.39–0.81, $p = 0.002$) is more statistically significantly different than the division of "pl0 and pl1 vs. pl2" (HR = 0.59, 95% CI: 0.36–0.95, $p = 0.03$) (data not shown). Thus, the pl1 and pl2 patient groups were defined as the VPI group and their survival was compared with the pl0 group in the following analysis. The 5-year OS rates of non-VPI and VPI patients were 80.8% and 58.9%, respectively, with a statistically significant difference ($p < 0.0001$) (Table 1b). The current multivariate survival analysis showed that VPI is an independent prognostic factor (Table 2), consistent with previous studies showing VPI to be a strong independent factor for a poor prognosis in NSCLC patients.

Although it remains unclear why VPI is associated with a worse prognosis, one possible reason is that VPI may have strong lymph node involvement in lung cancer. The visceral pleura is very rich in lymphatic vessels, with an intercommunicating "network" arranged over the lung surface and penetrating into the lung parenchyma to join the bronchial lymph vessels with drainage to various hilar lymph nodes. The larger lymphatic vessels in the visceral pleura have one-way valves which also direct flow toward the hilum of the lung [19]. We observed that VPI was significantly associated with more extensive hilar or mediastinal lymph node involvement ($p < 0.001$, chi-square test), which is consistent with other studies [8,20]. The present findings support the suggestion by Shimizu et al. that there is a possible VPI tumor cell pathway through the subpleural lymphatics, hilar lymph nodes, and into the mediastinal lymph nodes [4]. Mizuno et al. reported that in the documented absence of pleural invasion, prognosis is similar

Table 3b
Proposal on incorporating VPI into T classification.

Group		7th edition T-factor (N0M0: stage)	Our proposal T-factor (N0M0: stage)
A	≤3 cm, non-VPI	T1a/T1b (stage IA)	T1a/T1b (stage IA)
B	≤3 cm, VPI	T2a (stage IB)	T2a (stage IB)
C	3.1–5 cm, non-VPI	T2a (stage IB)	T2a (stage IB)
D	3.1–5 cm, VPI	T2a (stage IB)	**T2b (stage IIA)**
E	5.1–7 cm, non-VPI	T2b (stage IIA)	T2b (stage IIA)
F	5.1–7 cm, VPI	T2b (stage IIA)	T2b (stage IIA)

T-factor and stage in bold in our proposal differ from those in the 7th edition classification.
Non-VPI, without visceral pleural invasion; VPI, with visceral pleural invasion. Any pN, patients regardless of lymph node metastasis; pN0, patients without lymph node metastasis; 5-y OS, 5-year overall survival rate.