dVS yielded results similar to those of standard surgical procedures, at least in the perioperative period, and it appears safe, reliable, and less invasive. This instrument is better for the treatment of tumors located in difficult-to-reach areas than conventional VATS, but the positioning of all units and the location of arm ports need suitable directional settings, which depend on the tumor location.

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## Conflicts of interest statement

None declared.

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ORIGINAL ARTICLE

# Do tumours located in the left lower lobe have worse outcomes in lymph node-positive non-small cell lung cancer than tumours in other lobes?

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## Abstract

**OBJECTIVES**: Although an association between prognosis and lobar location of lung cancer, particularly the left lower lobe (LLL), has been suggested, the certainty of such association remains controversial. The purpose of this study was to evaluate the impact of tumour lobar location on surgical outcomes as an independent prognostic factor for survival in our non-small cell lung cancer (NSCLC) patient series.

**METHODS**: We retrospectively reviewed 978 NSCLC patients who underwent complete resection in our hospital between 2000 and 2007. We statistically analysed the association between clinicopathological factors and clinical outcomes.

**RESULTS**: Among the 978 patients reviewed, the NSCLC was located in the LLL in 143 (14.6%) patients, and lymph node involvement was identified in 210 patients (21.5%). The 5-year overall survival rates of patients whose NSCLC was located in the LLL and in other lobes (non-LLL) were 73.1 and 74.3%, respectively, and showed no significant association ($P = 0.86$). On the other hand, the 5-year survival rates of patients whose NSCLC occurred in the LLL ($n = 33$) and non-LLL ($n = 177$) and with lymph node metastasis were 32.7 and 57.7%, respectively, and showed a significant association ($P = 0.01$). Therefore, we performed a more detailed analysis on the 210 NSCLC patients with lymph node metastasis. On multivariate analysis, we found that LLL tumour ($P = 0.02$), tumour size >3 cm ($P = 0.02$) and N status ($P < 0.001$) were significant independent predictors for survival.

**CONCLUSIONS**: LLL tumours with lymph node metastasis are strongly associated with mortality in NSCLC patients. The location of the primary tumour may contribute in determining the optimal management strategy and accurate prediction of prognosis.

**Keywords:** Lung cancer • Prognostic factor • Tumour location • Lymph node metastasis

## INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the world, and non-small cell lung cancer (NSCLC) comprises the majority of lung cancers. 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].

Lower lobe tumours have been reported to be associated with poorer survival than upper lobe tumours [4]. On the other hand, it has also been contended that tumour location within the lungs does not independently predict survival [5]. Thus, whether the location of a tumour in the lungs is a prognostic factor remains controversial.

The purpose of this study was to evaluate the impact of tumour lobar location as an independent prognostic factor for survival in a long series of NSCLC patients at our institution.

## MATERIALS AND METHODS

From January 2000 to December 2007, a total of 1145 patients underwent complete resection of NSCLC at our hospital. We excluded 167 patients 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 978 patients were enrolled in this study. We defined complete resection as lobectomy or more extensive lung resection with systemic ipsilateral hilar and mediastinal lymph node dissection and no evidence of residual cancer either macroscopically or microscopically. The median follow-up period was 3.8 years. The Institutional Review Board of our hospital approved the data collection and analyses, and waived the need to obtain written informed consent from each patient since all data were retrospective.

We reviewed the medical records of each patient for clinicopathological information including age, gender, pathological

nodal involvement, vessel invasion (vascular invasion and lymphatic permeation), pleural invasion (as defined in the 7th Edition of the TNM Classification for Lung and Pleural Tumors), tumour 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. The histologic type was determined according to the 3rd Edition of the World Health Organization Classification. We used haematoxylin–eosin and Elastica van Gieson stains for the evaluation of vessel and pleural invasion.

Overall survival (OS) was estimated using the Kaplan–Meier method, and differences in survival were determined by log-rank analysis. Zero time was the date of pulmonary resection, and the end point was defined as the date of death from any cause. The last follow-up observation was censored when the patient was alive or lost to follow-up. All *P*-values were two-sided and *P*-values <0.05 were considered a statistically significant difference. Univariate analysis was conducted among the different groups.

Categorical variables were analysed using the $\chi^2$-test. Differences between two groups were tested using Student's *t*-test. Multivariate analysis was performed using the Cox proportional hazards model to examine the association between survival and potential prognostic factors. A probability value of <0.05 was considered statistically significant. All statistical calculations were performed using SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

The characteristics of the patients are shown in Table 1. The 978 patients in this study consisted of 574 men (58.7%) and 404 women (41.3%). The mean age was 65.0 years (range: 22–86). Among the 978 patients, the tumours in 143 (14.6%) patients were located in the LLL. The 143 patients with LLL tumours had a lower proportion of adenocarcinoma than the 835 patients with non-LLL tumours, and there were significant differences in histology (adenocarcinoma vs non-adenocarcinoma) between LLL and non-LLL tumours ($P = 0.02$). Tumour size was significantly larger in LLL tumours than in non-LLL tumours (mean: 3.4 and 2.9 cm, respectively; $P = 0.0001$). However, there was no

**Table 1:** Patient characteristics ($n = 978$)

| Variable | Number (%) | | | *P*-value (LLL vs non-LLL) |
|---|---|---|---|---|
| | All ($n = 978$) | LLL ($n = 143$) | Non-LLL ($n = 835$) | |
| Sex (%) | | | | |
| Men | 574 (58.7) | 85 (59.4) | 489 (58.6) | 0.84 |
| Women | 404 (41.3) | 58 (40.6) | 346 (41.4) | |
| Mean age (range) | 65.0 (22–86) | 66.0 (22–85) | 64.8 (22–86) | 0.19 |
| Tumour location (%) | | 143 (14.7) | RUL 340 (34.8)<br>RML 69 (7.1)<br>RLL 212 (21.7)<br>LUL 214 (21.9) | - |
| Histological type (%) | | | | |
| Adenocarcinoma | 726 (74.2) | 93 (65.0) | 633 (75.8) | 0.02* |
| Squamous cell carcinoma | 183 (18.7) | 38 (26.6) | 145 (17.4) | |
| Large cell carcinoma | 50 (5.1) | 7 (4.9) | 43 (5.1) | |
| Others | 19 (2.0) | 5 (3.5) | 14 (1.7) | |
| Operation procedure (%) | | | | |
| Lobectomy/bilobectomy | 961 (98.3) | 138 (96.5) | 823 (98.6) | 0.08 |
| Pneumonectomy | 17 (1.7) | 5 (3.5) | 12 (1.4) | |
| Mean tumour size (cm) (range) | 2.9 (0.4–15.0) | 3.4 (0.7–15.0) | 2.9 (0.4–13.0) | 0.0001* |
| pT factor (%) | | | | |
| T1a/T1b | 472 (48.3) | 69 (48.2) | 403 (48.3) | 0.85 |
| T2a/T2b | 441 (45.1) | 63 (44.1) | 378 (45.3) | |
| T3/T4 | 65 (6.6) | 11 (7.7) | 54 (6.4) | |
| pN factor (%) | | | | |
| N0 | 768 (78.5) | 110 (76.9) | 658 (78.8) | 0.37 |
| N1 | 119 (12.2) | 22 (15.4) | 97 (11.6) | |
| N2 | 91 (9.3) | 11 (7.7) | 80 (9.6) | |
| P-stage (%) | | | | |
| IA | 420 (40.9) | 63 (44.0) | 357 (42.7) | 0.47 |
| IB | 286 (29.2) | 35 (24.5) | 251 (30.1) | |
| IIA | 118 (12.1) | 22 (15.4) | 96 (11.5) | |
| IIB | 49 (5.0) | 9 (6.3) | 40 (4.8) | |
| IIIA | 105 (10.7) | 14 (9.8) | 91 (10.9) | |
| Vascular invasion (%) | 383 (40.9) | 56 (39.2) | 327 (39.2) | 0.95 |
| Lymphatic permeation (%) | 453 (48.5) | 71 (49.6) | 382 (45.7) | 0.39 |
| Pleural invasion (%) | 250 (25.6) | 42 (14.9) | 208 (24.9) | 0.49 |

RUL: right upper lobe; RML: right middle lobe; RLL: right lower lobe; LUL: left upper lobe; LLL: left lower lobe.
*$P < 0.05$.

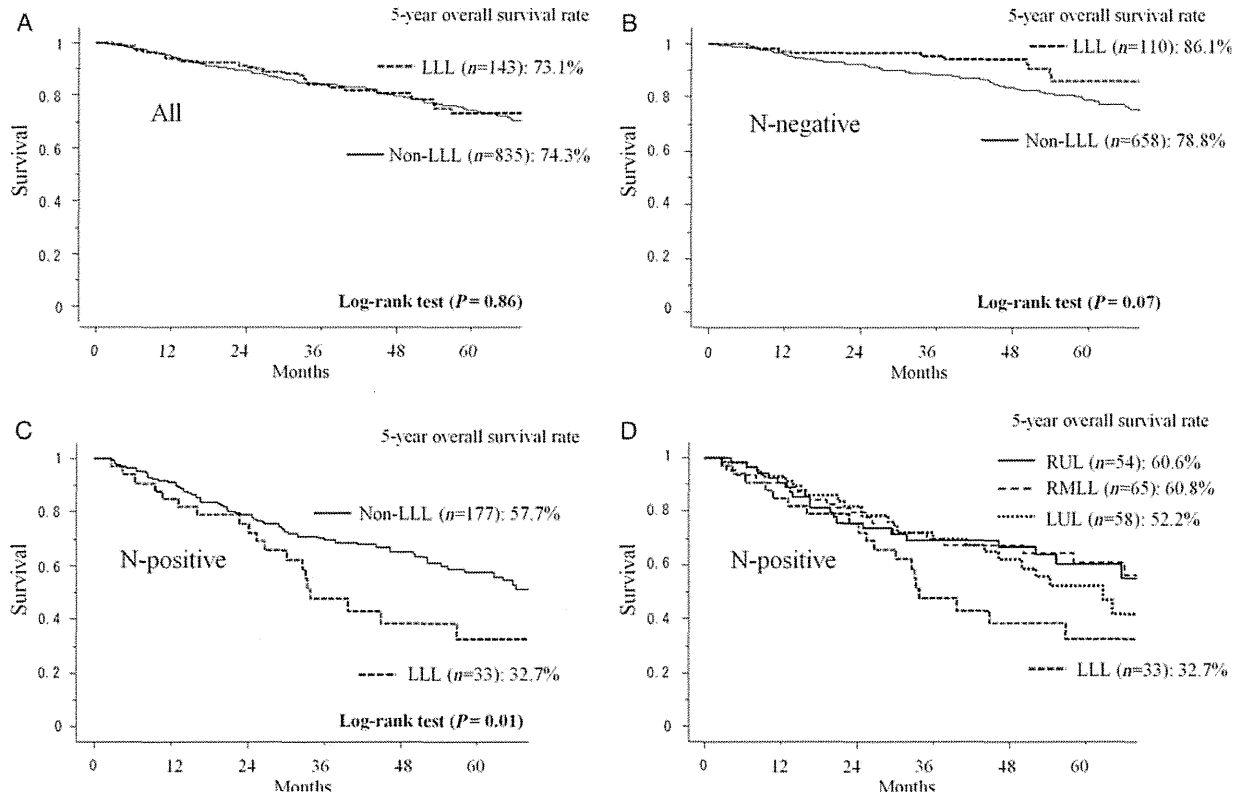


**Figure 1:** (**A**) Survival curves of all patients classified by the primary tumour site. No significant differences in survival outcomes were observed ($P = 0.86$). (**B**) Survival curves of all patients without lymph node metastasis classified by the primary tumour site. No significant differences in outcomes were observed ($P = 0.07$). (**C**) Survival curves of all patients with lymph node metastasis classified by the primary tumour site. Significant differences in outcomes were observed ($P = 0.01$). (**D**) Survival curves of all patients with lymph node metastasis classified by the primary tumour site. Significant differences in outcomes were observed between LLL and RUL, or RMLL ($P = 0.03$ and 0.03, respectively), and no slight differences in outcomes was observed between LLL and LUL ($P = 0.056$). RUL: right upper lobe; RMLL: right middle or lower lobe; LUL: Left upper lobe; non-LLL: other lobes; LLL: Left lower lobe.

significant difference between LLL and non-LLL tumours with regard to the several clinical factors including sex, age, operation procedure, pT factor, pN factor, p-Stage, vascular invasion, lymphatic permeation and pleural invasion.

The 5-year OS rates in patients whose NSCLC were located in the LLL and other lobes (non-LLL) were 73.1 and 74.3%, respectively ($P = 0.86$) (Fig. 1A). In 768 patients without lymph node metastasis, the 5-year OS rates for LLL ($n = 110$) and non-LLL ($n = 658$) tumours were 86.1 and 78.8%, respectively, with no significant difference ($P = 0.07$) (Fig. 1B). However, in 210 patients with lymph node metastasis, the 5-year OS rates for LLL ($n = 33$) and non-LLL ($n = 177$) tumours were 32.7 and 57.7%, respectively, showing a significant association between survival rates and tumour lobe location in NSCLC patients with nodal metastasis ($P = 0.01$) (Fig. 1C). Notably, although the 5-year OS rate of N1 patients with non-LLL (74.2%) was significantly higher than that of N1 patients with LLL (33.6%) ($P = 0.0007$), there was no significant difference in the 5-year OS rate between N2 patients with LLL (39.0%) and N2 patients with non-LLL (36.9%) ($P = 0.42$) (data not shown). Moreover, we divided all cases into four groups [right upper lobe (RUL), right middle or lower lobe (RMLL), left upper lobe (LUL) and left lower lobe (LLL)] according to tumour locations, and performed analysis for survival. In patients with lymph node metastasis, the 5-year OS rates for RUL ($n = 54$), RMLL ($n = 65$), LUL ($n = 58$) and LLL ($n = 33$) were 60.6, 60.8, 52.1 and 32.7%, respectively (Fig. 1D). There was a statistically significant difference in the 5-year OS rate between LLL and RUL, or RMLL ($P = 0.03$ and 0.03, respectively), but while the 5-year OS rate for LLL was worse than that of LUL there was no statistically significant difference ($P = 0.056$). On the other hand, in patients without lymph node metastasis, the 5-year OS rates for RUL ($n = 286$), RMLL ($n = 216$), LUL ($n = 156$) and LLL ($n = 110$) were 78.0, 78.5, 80.6 and 86.1%, respectively. There was no significant difference in the 5-year OS rates among LLL and RUL, RMLL, or LUL ($P = 0.16$, respectively) (data not shown).

We conducted a more detailed analysis on the 210 patients with lymph node metastasis to clarify the association of various prognostic factors with survival rate. Univariate analysis showed that a tumour size of >3 cm ($P = 0.01$), N2 ($P < 0.0001$), the presence of pleural invasion ($P = 0.006$) and LLL tumour ($P = 0.01$) were risk factors significantly associated with survival (Table 2). Multivariate analysis using the Cox regression model demonstrated that LLL tumour [hazard ratio (HR) = 1.84, 95% confidence interval (CI): 1.12–3.05, $P = 0.02$], a tumour size of >3 cm (HR = 1.68, 95% CI: 1.08–2.63, $P = 0.02$) and N status (HR = 2.28, 95% CI: 1.47–3.54, $P < 0.001$) were significant independent predictors for survival (Table 3).

**Table 2:** Univariate survival analysis in N-positive NSCLC ($n = 210$)

| Variable | | Number of patients | 5-year survival rate (%) | *P*-value |
|---|---|---|---|---|
| Sex | Men | 146 | 53.7 | 0.93 |
| | Women | 64 | 53.5 | |
| Age | <70 | 139 | 54.3 | 0.12 |
| | 70 | 71 | 51.6 | |
| Right vs left | Right | 119 | 60.5 | 0.08 |
| | Left | 91 | 44.9 | |
| Location | Non-LLL | 177 | 57.7 | 0.01* |
| | LLL | 33 | 32.7 | |
| Histological type | Non-Ad | 90 | 55.0 | 0.37 |
| | Ad | 120 | 52.6 | |
| Tumour size | 3 cm | 86 | 65.2 | 0.009* |
| | >3 cm | 124 | 45.4 | |
| N status | N1 | 119 | 65.7 | <0.0001* |
| | N2 | 91 | 36.6 | |
| Pleural invasion | Absent | 125 | 64.2 | 0.006* |
| | Present | 83 | 37.3 | |
| Vascular invasion | Absent | 45 | 67.6 | 0.06 |
| | Present | 153 | 47.2 | |
| Lymphatic permeation | Absent | 26 | 56.2 | 0.66 |
| | Present | 174 | 52.2 | |

LLL: left lower lobe; non-LLL: other lobes; Ad: adenocarcinoma; non-Ad: other histological types.
*$P < 0.05$.

**Table 3:** Multivariate survival analysis in N-positive NSCLC ($n = 210$)

| Variable | HR | 95% CI | *P*-value |
|---|---|---|---|
| Location (LLL vs non-LLL) | 1.84 | 1.12–3.05 | 0.02* |
| Tumour size (>3 cm vs 3 cm) | 1.68 | 1.08–2.63 | 0.02* |
| N status (N2 vs N1) | 2.28 | 1.47–3.54 | <0.001* |
| Pleural invasion (present vs absent) | 1.24 | 0.79–1.94 | 0.26 |

LLL: left lower lobe; non-LLL: other lobes; CI: confidence interval.
*$P < 0.05$.

## DISCUSSION

The TNM stage classification was developed to provide high specificity for patients with a similar prognosis and treatment options. There are several recent reports of poor prognostic factors in lung cancer [6, 7]. In the TNM classification, the location of the primary tumour does not affect prognosis. However, several studies of prognostic factors in resected NSCLC showed that lower lobe lesions increase mortality.

Lower lobe tumours were previously shown to be associated with worse-term survival than upper lobe tumours [4]. Moreover, death within 5 years is caused more frequently by lower lobe tumours than by upper lobe tumours, the difference between the right and left lungs being largely due to the low survival rate associated with tumours of the LLL [8]. A previous study has shown that differences in survival rates are associated with lobar location, such that patients with upper lobe tumours showed better long-term survival than patients with middle lobe or lower lobe tumours [9]. Recent studies have shown that tumours located in non-upper lobes such as the right middle lobe (RML), right lower lobe (RLL) and LLL independently had an increased risk of mortality in patients with stage IB NSCLC compared with tumours located in the upper lobes [10]. Similarly, multivariate analysis of patients undergoing surgery for T2 tumours previously showed that patients with LLL tumours had significantly worse survival outcomes than those who had tumours in other lobar locations [11]. Rocha *et al.* [12] found that the presence of the primary tumour in the lower lobe was the only statistically significant factor associated with upstaging, and other factors such as patient age, smoking history, weight loss, tumour size and tumour histology were not associated with upstaging.

In our NSCLC patient series, the OS curves between LLL and non-LLL patients were similar for all patients, and no significant difference was observed ($P = 0.86$). However, in patients with lymph node metastasis, the 5-year OS rates of LLL and non-LLL patients were 32.7 and 57.7%, respectively, showing a significant difference between the two categories ($P = 0.01$). Moreover, according to the analysis between the four groups of tumour locations in patients with lymph node metastasis, LLL tumours had significantly worst survival, with only a slight difference between LLL and LUL ($P = 0.056$). When we subdivided the N category into pN1 and pN2 subgroups, the survival rate of LLL patients was significantly different from that of non-LLL patients for pN1 ($P < 0.001$), but not for pN2 ($P = 0.42$). Moreover, the 5-year OS of 33.6% in LLL patients with N1 was lower than that of 39.0% in LLL with N2, but there was no statistically significant difference ($P = 0.39$) (data not shown). In the N1 patients, there was no significant difference in the number of metastatic lymph node, in the pattern of recurrence (locoregional or distant), or in the cause of death between LLL and non-LLL groups (data not shown). We have not fully clarified the underlying reasons for this finding but we speculate that the lack of statistical significance may be, in part, due to the small number of LLL patients with pN2 ($n = 11$). These results suggest that the unfavourable prognosis of lower lobe lesions is affected by the presence or absence of lymph node metastasis. In particular, this study showed that tumour size ($P = 0.01$), N status ($P < 0.0001$), pleural invasion ($P < 0.01$) and tumour location ($P = 0.01$) accounted for significant differences between the OS curves of patients with lymph node metastasis. On multivariate analysis, LLL tumour with lymph node metastasis was shown to be an independent prognostic factor for OS, as well as tumour size and N status (HR = 1.84, 95% CI: 1.12–3.05, $P = 0.02$).

Right lung cancer spreads mainly to mediastinal ipsilateral nodes. In left lung cancer, contralateral and ipsilateral lymphatic spread occurs with the same frequency on each side [8]. The lymphatic drainage of the lungs to the mediastinal lymph nodes has been studied extensively. Various techniques of injection of dyes into the lymphatic channels of lungs from autopsy specimens of stillborn infants and adults without pulmonary disease have been used by Rouvière [13]. Notably, the dynamic study using lymphoscintigraphy in normal healthy subjects and the lymphatic drainage routes described by Hata *et al.* [14] are generally in agreement with the patterns described by Nohl-Oser [15]. Drainage from the basal segments of the RLL rarely distributed to the left side of the mediastinum. In contrast, contralateral mediastinal drainage from the left lung is relatively common,

**Table 4:** Summary of studies of the association between tumour location and prognosis

| Authors | Journal | N | Case | Location | *P*-value |
|---|---|---|---|---|---|
| Positive association | | | | | |
| Iwasaki *et al.* [11] | *ICVTS* 2004 | 93 (total, 268) (34.7%) | T2 | LLL vs others | 0.0258 |
| Ichinose *et al.* [9] | *JTCVS* 2001 | N2 | Stage IIIA-N2 | Four primary sites (LLL, RMLL, RUL, LUL) | 0.0378 |
| Ou *et al.* [10] | *Cancer* 2007 | N0 | Stage IA/IB | Upper vs non-upper | 0.0072/<0.0001 |
| Bignall and Moon [8] | *Thorax* 1955 | 133 (total, 233) | Unknown | Four primary sites (right upper/lower, left upper/lower) | Data not shown (LLL: worst survival) |
| Hayakawa *et al.* [18] | *JJCO* 1996 | 126 (total, 141) | Stage III(A/B)s | Upper + superior segment of lower lobe vs lower lobe | 0.032 |
| Inoue *et al.* [19] | *JTCVS* 2004 | N2 | Stage IIIA | Upper vs middle or lower | 0.0362 |
| Negative association | | | | | |
| Puri *et al.* [5] | *ATS* 2010 | 0/144 (total, 841) | Stage I/II | Upper vs lower, right vs left | 0.57/0.63, 0.78/0.71 |
| Inoue *et al.* [19] | *JTCVS* 2004 | N0, 1 | StageIIIA-N2 (N0–1 data are only shown in discussion) | Upper vs middle or lower | No survival difference (data not shown) |
| Huhti *et al.* [20] | *EJRD* 1983 | NX | Unknown | Four major lobes | Data not shown |

LLL: left lower lobe; RMLL: right middle and lower lobe; RUL: right upper lobe; LUL: left upper lobe.

occurring most frequently through the subcarinal nodes, as initially pointed out by Rouvière [11] and reconfirmed by all subsequent studies. Occasionally, crossover occurs through the lower paratracheal nodes in drainage from the LLL. Nohl-Oser [15] previously reported that the metastasis rate to the right upper paratracheal nodes was 22% for LUL cancer and 40% for LLL cancer, and the incidence rate of contralateral metastases from tumours in the RLL in metastatic mediastinal node disease was 7%. Left hilar lymphatics connect to left tracheobronchial nodes or right paratracheal nodes via subcarinal nodes. Therefore, LLL tumours may tend to spread to contralateral mediastinal lymph nodes.

Although Toker *et al.* [16] reported that dissection of the contralateral paratracheal lymph nodes is possible without undertaking more extensive surgical dissection, including cervical dissection techniques, in our NSCLC patient series, we performed systematic mediastinal lymph node dissection, defined as the en-bloc removal of all ipsilateral lymph nodes along with the surrounding fat tissue. Since we did not perform contralateral mediastinal lymph node dissection, we could not accurately evaluate the involvement of contralateral mediastinal lymph nodes in patients with no CT evidence of contralateral lymph nodes. Patients with LLL tumours with lymph node metastasis may include N3 patients who have contralateral mediastinal lymph node metastasis, despite no evidence of contralateral lymphadenopathy on preoperative CT film. In such cases, the majority of first recurrences may be locoregional. In our patient series, however, the first recurrence was more frequently distant recurrence than locoregional recurrence in both LLL and non-LLL tumours, and there were no significant differences in recurrence rates between LLL and non-LLL tumours (data not shown). Distant recurrence is easier to assess clinically and radiographically than locoregional recurrence, and thus the rate of locoregional recurrence can be underreported. Lymph node metastasis promoted a higher incidence of distant recurrence rather than local recurrence [17].

Moreover, tumour size was significantly larger in LLL tumours than in non-LLL tumours ($P = 0.0001$). These patients with LLL tumours had worse survival outcomes, possibly due to the LLL tumours being hidden by the shadow of the left heart, which made them difficult to find.

A summary of studies [5, 8–11, 18–20] showing the association between tumour location and prognosis is shown in Table 4. Regardless of LLL tumours, most studies, except the study of Ou *et al.* [8], indicate that in the advanced stage, tumour location affects the prognosis, Puri *et al.* [5] showed no relationship between tumour location and prognosis in the early stage and Inoue *et al.* [19] reported that tumour location does not affect prognosis in N0 or N1 tumours. Based on our present results, we speculate that tumour location is less likely to affect survival in early-stage tumours, but the prognosis may be affected by tumour location in advanced stage tumours. We, therefore, conclude that LLL tumours have worse outcomes in lymph node-positive NSCLC.

The limitations of this study include its retrospective nature, which evaluated cases from 2000, a small sample size, and the fact that routine adjuvant chemotherapy for N1 or higher patients was started in 2004. These limitations complicated the evaluation of the effects of tumour location on prognosis with respect to adjuvant chemotherapy.

In conclusion, LLL tumours were found to be strongly associated with mortality in NSCLC patients with lymph node metastasis. The location of the primary tumour may aid in determining the optimal management strategy and allow for more accurate prediction of prognosis. This may affect staging criteria and further studies are needed to clarify the underlying reasons why LLL tumours with lymph node metastasis have unfavourable prognoses.

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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.



## 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′-AAAGGAUUCUGCUGUCGGUCCCACU-3′) and antisense (5′-UGGUGGGCACAGAAUCUGGACU-3′); has-miR-542-5p sense (5′-UCGGGGAUCAUCAUGUCAUGAGA-3′) and antisense (5′-ugugacagauugauaacugaaa-3′) miR-nontarget control miRNA sense (5′-AUCCGCGCGAUAGCACGUAUU-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′-GTGACCGTTTGGGAGTTGATGA-3′ and reverse primer, 5′-GGCTGAGGGAGGCGTTCTC-3′.

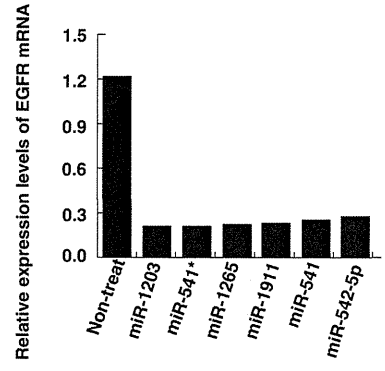
### 2.4. Immunohistochemistry and in situ hybridization

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




C

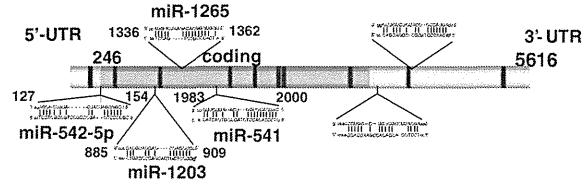



**Fig. 1.** Identification of miRNAs that target EGFR using 96-well plate transfections. (A) Candidate miRNAs targeting the EGFR sequence on a 96-well plate. (B) Down-regulation of EGFR mRNA by synthetic miRNA. Real-time PCR showed that synthetic miR-1203, miR-541*, miR-1265, miR-1911, miR-541, and miR-542-5p 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.

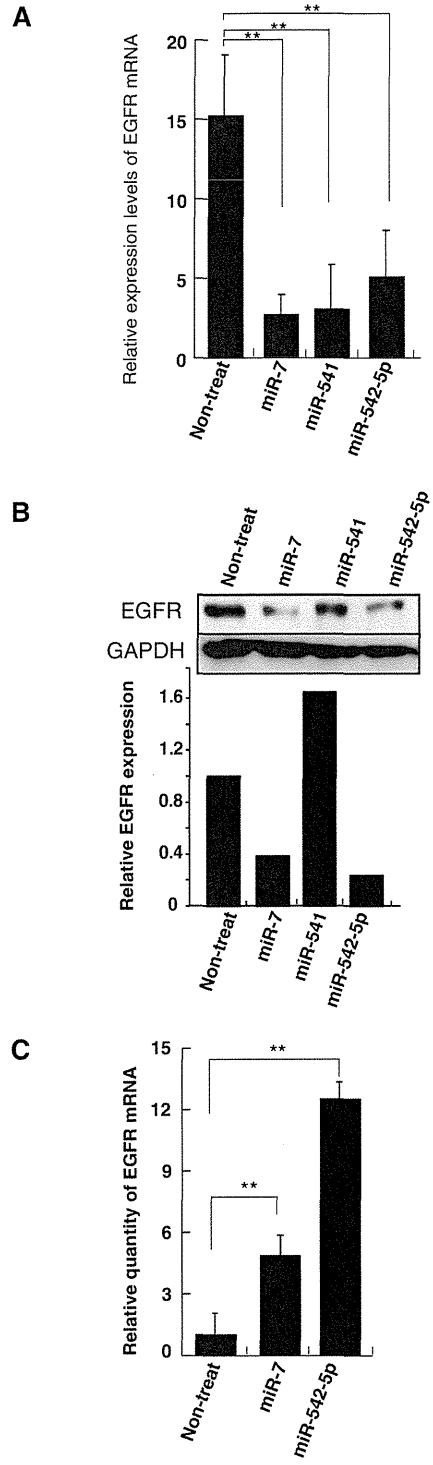



**Fig. 2.** EGFR is a direct target of miR-542-5p in lung cancer cells. (A) Quantitative analysis of EGFR mRNA by real-time PCR. EGFR mRNA significantly decreased upon transfection of synthetic miR-7, miR-541, and miR-542-5p in A549 cells. *Bars*, mean ± SD. $^{**}P < 0.02$ (B) Regulation of EGFR protein 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.

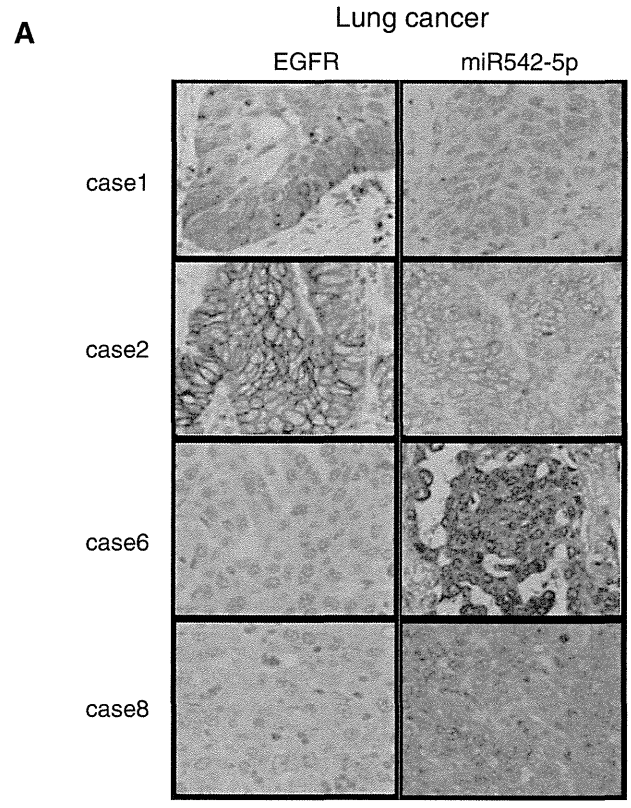
## 3. Results

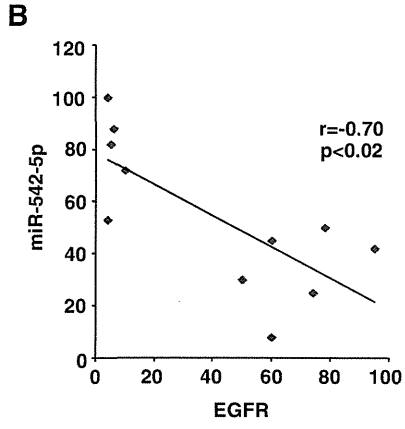
### 3.1. Identification of EGFR target miRNA

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 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.

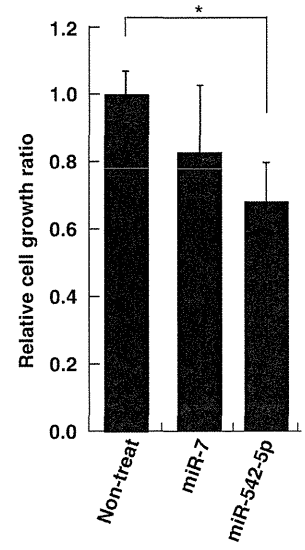



**Fig. 4.** miR-542-5p modulates the proliferation of human lung cancer cell lines. We determined the number of A549 cells 72 h after transfection with the amiR-7 or miR-542-5p by MTT assay. *Bars*, mean ± SD. (*$P < 0.05$).

## 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

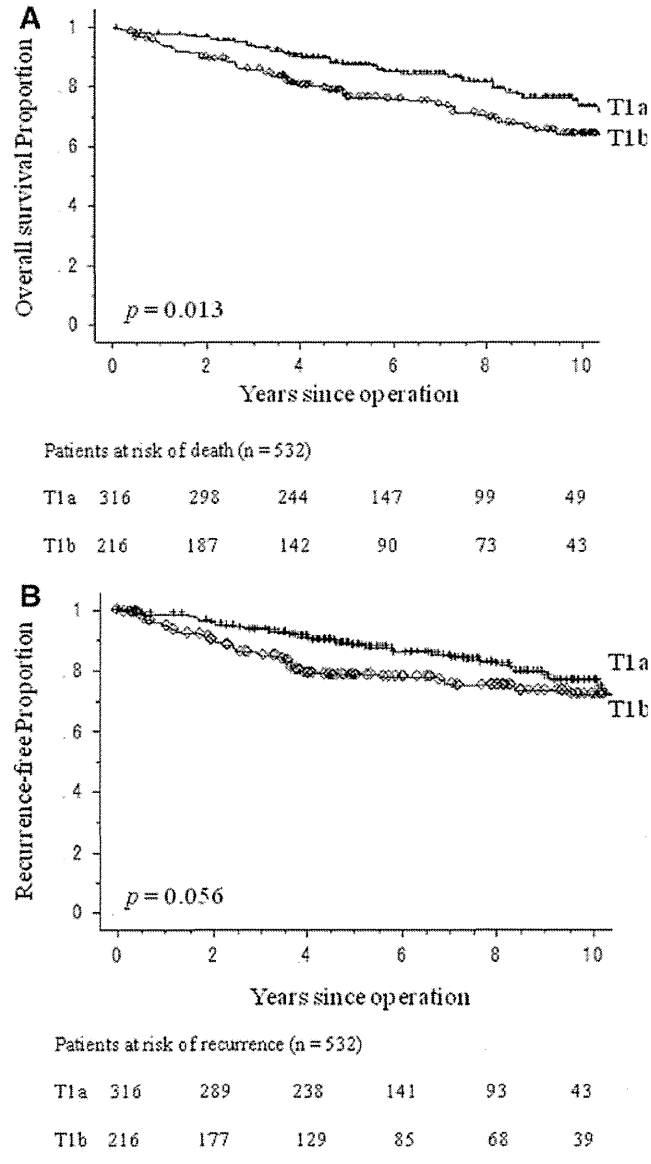


**FIGURE 1.** *A*, Overall survival curves of patients with T1a or T1b disease. *B*, Recurrence-free proportion curves of patients with T1a or T1b disease.

$\chi^2$ 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 |

**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

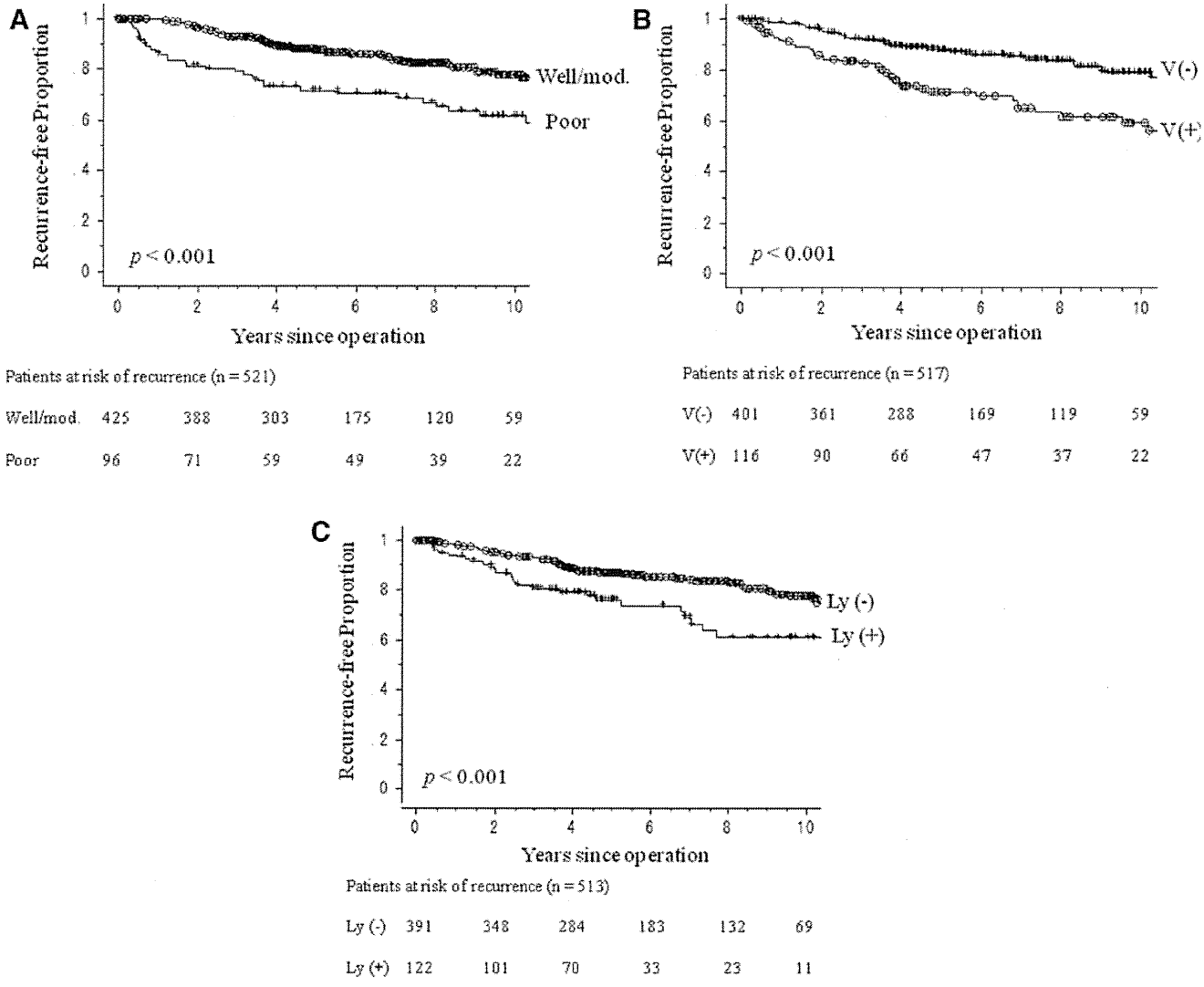


**FIGURE 2.** *A*, Recurrence-free proportion curves according to tumor differentiation. *B*, Recurrence-free proportion curves according to blood-vessel invasion. *C*, Recurrence-free proportion curves according to lymph-vessel invasion.

**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

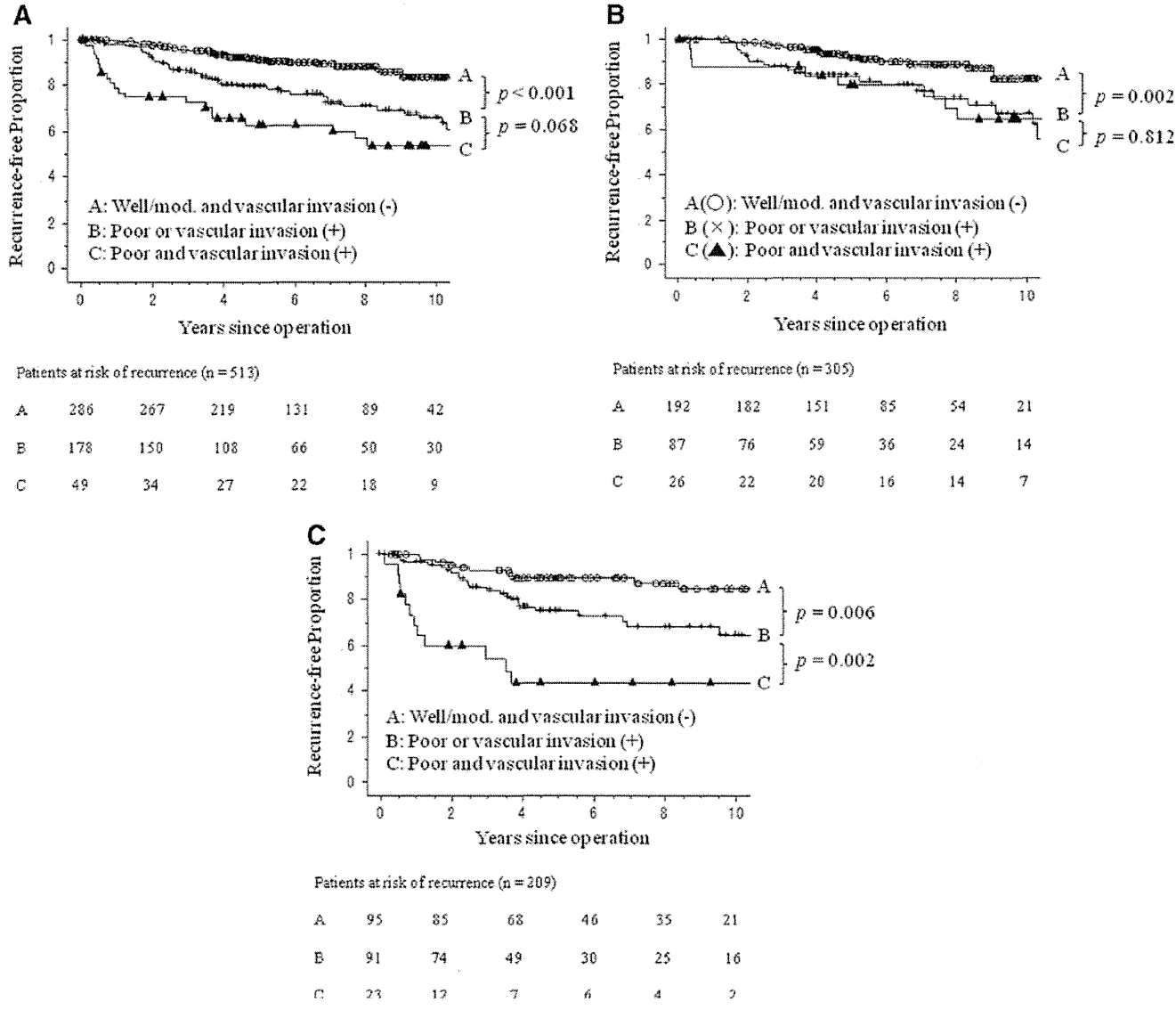


**FIGURE 3.** *A*, Recurrence-free proportion curves for all stage IA; *B*, T1a; and *C*, T1b patients with well- or moderately differentiated carcinoma and no vascular invasion (curve A), poorly differentiated carcinoma or vascular invasion (curve B), and both poorly differentiated carcinoma and vascular-invasion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