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Article

# Ki-67 Labeling Index Is Associated with Recurrence after Segmentectomy under Video-assisted Thoracoscopic Surgery in Stage I Non-small Cell Lung Cancer

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**Objectives:** Video-assisted thoracoscopic surgery (VATS) segmentectomy for small or early stage non-small cell lung cancer (NSCLC) remains controversial. Here, we investigated the clinical importance of predicting recurrence by Ki-67 in VATS segmentectomy for stage I NSCLC.

**Methods:** In a retrospective study, 44 consecutive patients in p-stage I underwent VATS segmentectomy between September 2003 and April 2009. After clinicopathological factors were compared with Ki-67 expression, the relationship between Ki-67 labeling indexes (LI) or mRNA expression by quantitative RT-PCR and prognosis was investigated.

**Results:** Five of 44 VATS segmentectomy patients relapsed. In the relapsed patients, 3 (6.8%) were local recurrences and 2 (4.5%) were distant metastases. There was no significant difference between clinicopathological factors and recurrence; however, patients with Ki-67 LI less than 5% showed better disease-free survival than patients with Ki-67 LI over 5% ( $p = 0.04$ ). In multivariate Cox regression analysis, although there was no significantly different in disease-free survival by age, histology, tumor size, only Ki-67 LI showed a significant prognostic factor of recurrence (HR = 12.5, 95% CI = [1.1-1407],  $p = 0.04$ ).

**Conclusions:** Ki-67 LI after VATS segmentectomy was a prognostic factor of disease-free survival in NSCLC and the treatment of choice for patients with positive LI may be considered, in addition to adjuvant chemotherapy, or lobectomy.

**Key words:** Ki-67, VATS, lung cancer, segmentectomy, stage I

## Introduction

Non-small cell lung cancer (NSCLC) represents approximately 80% of all lung cancers. Although stage I

NSCLC patients undergo radical surgery, 30% to 60% will develop recurrence and die as a result of their disease.<sup>1)</sup>

The only randomized trial that compared lobectomy and sublobar resection of the lung was reported in 1995 by the Lung Cancer Study Group (LCSG)<sup>2)</sup> and showed that sublobar resection had the potential for local recurrence; thereafter, the standard treatment for stage I non-small cell lung cancer was the lobectomy. However, the LCSG study might have been misleading because a number of wedge resections were included in the sublobar resection group. As recently reported, sublobar resection showed an equivalent outcome to lobectomy regarding

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the prognosis and local recurrence in small NSCLC, if patient selection for sublobar resection was adequate.<sup>3)</sup>

Video-assisted thoracic surgery (VATS) has been introduced in a variety of thoracic operations, including small-sized lung cancer. VATS is less invasive and provides benefits due to the rapid postoperative recovery and shortening the hospital stay.<sup>4)</sup> We have reported that VATS segmentectomy for early stage lung cancer is a good surgical option when the patient is well-selected<sup>5, 6)</sup>; however, VATS sublobar resection as definitive management for early stage lung cancer remains controversial and might be associated with increased local recurrence rates.

Heterogeneity of tumor biology leads to a variety of prognoses or recurrences due to metastatic or invasive behavior. Several markers to predict the risk of recurrence have been reported in NSCLC.<sup>7)</sup> The expression of Ki-67, which is a marker of proliferative activity, is important to stratify the group of high-risk patients.<sup>8)</sup> The purpose of this study was to evaluate the possibility of Ki-67 as a predictor of recurrence after VATS segmentectomy under the hypothesis that VATS segmentectomy is equivalent to lobectomy in stage I NSCLC.

## Materials and methods

### Patients

In a retrospective study, VATS segmentectomy was performed in 44 consecutive stage I patients at Oita University Hospital (Oita, Japan) between September 2003 and April 2009. The study was approved by the Institutional Review Board of our hospital and all patients completed informed consent forms. Segmentectomy was accompanied with systemic radical lymphadenectomy. All patients were operated with curative intent using VATS.

Clinically, T1 N0 M0 for segmentectomy was eligible in this retrospective analysis, and final pathological stage IA and IB was analyzed in this study. Segmentectomy was intended on the basis of tumor size < 20 mm, and segmental margin > 10 mm. bi- or tri-segmentectomy (multi-segmentectomy) or lobectomy was followed when the segmental margin was less than 1 cm.

Postoperative pathological findings were made by two pathologists and final staging and histology were evaluated according to the UICC TNM classification (Sixth edition). Postoperative follow-up was practiced every 3 months by blood tests, chest x-ray, and CT scan at 6-month intervals. If any finding of relapse was observed,

the patients underwent subsequent radiotherapy and/or chemotherapy. Re-operation was one of the options to treat local recurrence.

### Surgical technique

Our surgical technique is similar to previous reports<sup>6)</sup>; if necessary, a utility access port of 3–4 cm in length was added to the 4 ports. All procedures were performed by visualization using a television monitor, so-called complete VATS. The intrathoracic procedure regarding hilar and intralobar vessel treatment was the same in both segmentectomy and lobectomy; however, the difference was segmental plane management by electrocautery or stapler. Theoretically, the segmental artery is divided and ligated or stapled first, following pulmonary vein ligation, stapling or bronchus stapling. If the intraoperative examination of hilar or intralobar lymph nodes by frozen section showed metastases, segmentectomy was converted to lobectomy. Both procedures were followed by systemic mediastinal lymph node dissection.

### Immunohistochemical analysis

Four micrometer sections were prepared for tissue slides. Antigen retrieval was performed at 121°C for 15 minutes in an autoclave with citrate buffer (pH9.0) after deparaffinization. Ten percent goat serum (Nichirei Tokyo, Japan) was used to block nonspecific binding. Staining with polyclonal anti-Ki-67 antibody (DAKO, Glostrup, Denmark) with diluents, 1 : 100, was performed overnight at 4°C. After a reaction with 3% hydrogen peroxide for 20 minutes at room temperature, polymer anti-mouse antibody (K4002; Dako Glostrup, Denmark) for Ki-67 was applied and incubated for 30 minutes at room temperature. Negative controls were incubated without the primary antibody.

IHC staining (labeling index) was evaluated as previously described.<sup>9)</sup> The numbers of Ki-67 positive cells were counted in the 200 tumor cells and 5% is the cut-off between high and low expression. For reliability of evaluation, two independent doctors (K.T and S. Y) estimated positivity by serial two sections staining.

### Quantitative PCR analysis

RNA was extracted by RNeasy FFPE kit<sup>TM</sup> according to the manufacturer's manual (QIAGEN, Tokyo, Japan). For quantitative evaluation of the RNA expression by PCR, we used Taqman PCR methods (TaqMan<sup>®</sup> Gene Expression Assays; Applied Biosystems, Tokyo, Japan) as previously reported.<sup>10)</sup> The Ki-67 gene was amplified by

**Table 1** Characteristics of the patients

Characteristics	Total	Ki-67 LI $\geq$ 5%	p	Ki-67 mRNA overexpression	p
Age < 70	24	5	0.17	8	0.91
$\geq$ 70	20	8		7	
Sex Male	25	11	0.02	8	0.74
Female	19	2		7	
Histological type					
Ad	10	3	0.06	4	0.4
Sq	7	5		2	
BAC	23	4		8	
Others	4	1		1	
Tumor size					
< 2cm	33	8	0.18	10	0.36
$\geq$ 2cm	11	5		5	
Lymphatic invasion					
(-)	39	9	0.02	13	0.97
(+)	5	4		2	
Vascular invasion					
(-)	42	12	0.51	14	0.64
(+)	2	1		1	
p-stage					
IA	41	12	0.88	14	0.98
IB	3	1		1	

BAC, bronchiolo-alveolar carcinoma; Sq, squamous cell carcinoma; Ad, adenocarcinoma; LI, labeling index

the following primer set: forward: ggtgtgcagaaaatccaaga, reverse: actgtccctatgacttctggtg.

The Ki-67 gene internal probe was the universal probe library probe #73 (Roche, Mannheim, Germany). The PCR amplification condition was one cycle at 50°C for 2 min, and 95°C for 10 min followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. The measured value was calculated by comparative Ct methods<sup>10)</sup> and GAPDH gene amplification was used as a control. All reactions were duplicated. The amounts of Ki-67 mRNA were expressed as n-fold GAPDH mRNA and the levels were compared relative to the normal lung tissues pool. A cut-off value of Ki-67 mRNA expression greater than 0.33, which was the value of the normal sample pool, was identified as a positive expression, and a lower value as negative.

### Statistical analysis

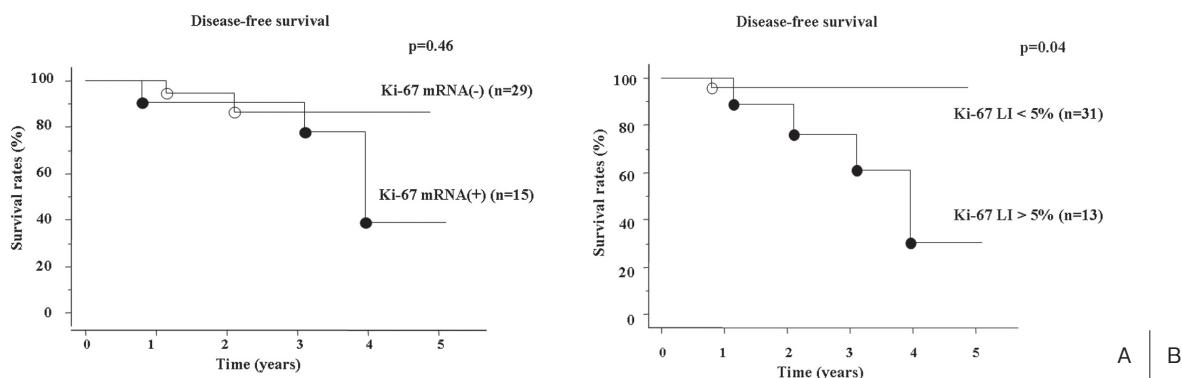
All statistical analysis was performed using StatView J5.0 (SAS Institute Inc., Tokyo, Japan). The different variables of tumors and normal tissues were analyzed with the chi-square test or Fisher's exact test. Time to progression was analyzed using the Kaplan-Meier method and

evaluated by the log-rank test. Statistically significant differences were accepted at  $p < 0.05$ .

## Results

### Clinicopathological relationships

Forty-four patients with stage I NSCLC underwent complete VATS segmentectomy between September 2003 and April 2009 in our hospital. Thirty-day and in-hospital mortality was 0%. Five of 44 patients who underwent VATS segmentectomy developed recurrence. Nineteen tumors occurred in the right lung and 25 in the left lung. A resected segment is as follows; S6 and basal segmentectomy were 5 and 2 in the right lung. Eight upper divisions, 2 lingular segments, 6 S6 segments, and 4 of apicodorsal and 2 basal segments were in the left lung. The relationship between clinicopathological factors and Ki-67 LI or mRNA expression is shown in **Table 1**. There was no relationship between age, histological type, tumor size, vascular invasion, pathological stage, and Ki-67 LI. However we found the significant correlation between Ki-67 LI and gender or lymphatic invasion. Ki-67 mRNA expression was not related to any other factors.



**Fig. 1**

- A:** Disease-free survival according to Ki-67 mRNA expression after VATS segmentectomy in stage I NSCLC. Open circle represents low expression of Ki-67 mRNA and closed circle is high expression. Statistical analyses were performed using the log-rank test.
- B:** Disease-free survival according to Ki-67 RI. Open circle represents Ki-67 LI less than 5% and closed circle is Ki-67 LI over than 5%. Five-year disease-free survival was significantly different between groups ( $p = 0.04$ ).

### Local recurrence, distant metastases and disease-free survival according to Ki-67 LI

44 VATS segmentectomy patients relapsed during the follow-up period (median 24.8 months); 3 (6.8%) were local recurrences and 2 (4.5%) were distant metastases. One patient died due to cancer relapse after segmentectomy. The 5-year overall survival rate after VATS segmentectomy was 95.6%. No significant differences between recurrence and Ki-67 LI were found in these events. Although there was no significant difference in disease-free survival by Ki-67 mRNA expression (**Fig. 1A**), disease-free survival in patients with Ki-67 LI  $< 5\%$  was significantly better than when  $\geq 5\%$  (**Fig. 1B**). The concordance rates were 73% between mRNA expression by RT-PCR and protein expression by IHC. Only Ki-67 LI positive or mRNA overexpression was 11% and 16% respectively. The 5-year disease-free survival rates were 96% in Ki-67  $< 5\%$  and 30% in Ki-67  $\geq 5\%$  (log-rank test,  $p = 0.04$ ). In multivariate Cox regression analysis, although there was no significantly different in disease-free survival by age, histology, tumor size, only Ki-67 LI showed a significant prognostic factor of recurrence (HR = 12.5, 95% CI = [1.1-140.7],  $p = 0.04$ , **Table 2**).

### Discussion

In previous reports, Ginsburg et al. first showed the superiority of lobectomy to sublobar resection for stage I NSCLC.<sup>2)</sup> The finding of the Lung Cancer Oncology Group study was a three-fold increase in local recurrence in patients who received sublobar resection; however,

**Table 2** Multivariate Cox proportional hazard model

haracteristics	HR	95% CI	p
Age ( $< 70$ vs $\geq 70$ )	1.7	0.17–16.9	0.66
Histology (BAC vs others)	4.1	0.2–86.2	0.36
Tumor size ( $< 2$ cm vs $\geq 2$ cm)	0.1	0.01–2.2	0.15
Ki-67 LI ( $< 5\%$ vs $\geq 5\%$ )	12.5	1.1–140.7	0.04

HR, hazard ratio; CI, confidence interval; BAC, bronchiolo-alveolar carcinoma; LI, labeling index

recent reports showed that anatomical segmentectomy provided feasible results in the recurrence rates and overall survival if adequate selection (stage IA and IB) was performed for NSCLC.<sup>11, 12)</sup> Okada et al. compared the results of anatomical segmentectomy to lobectomy in stage IA NSCLC.<sup>13)</sup> The 5-year survival of 87.1% in the segmentectomy group was favorable against 87.7% in the lobectomy group. The other group reported no difference between segmentectomy and lobectomy in patients with tumors  $< 2$  cm in diameter.<sup>14)</sup> These results support segmentectomy as a surgical option to cure small NSCLC. In our study, the 5-year survival rates of VATS segmentectomy were similar to a previous report.<sup>13)</sup>

Many factors influencing the outcome of survival and recurrence in segmentectomy have been proposed, such as lymph node dissection or sampling, and surgical margins from the tumor.<sup>15, 16)</sup> Several reports revealed that the distance from the tumor to the resected margin was a

good predictor of local recurrence.<sup>17)</sup> One centimeter margins were proposed or the margin distance was greater than the maximum diameter of the tumor.<sup>16)</sup> Even if the margin distance was good enough to perform a segmentectomy and was < 2 cm in diameter, we found 5 recurrences in our study. Tumor size is not a prognostic factor for recurrence because small-sized lung cancers were operated by segmentectomy. Therefore, we investigated the possibility of Ki-67 LI to predict recurrence in VATS segmentectomy. Ki-67 is a well-known cell proliferation marker and it is reported that the expression of this protein is a prognostic factor of disease-free survival in NSCLC.<sup>8)</sup> Ki-67 LI is also reported as a predictor of recurrence in stage IA NSCLC.<sup>9)</sup> Our study showed that Ki-67 LI was a significant correlation with lymphatic invasion, furthermore, and prognostic factor of disease-free survival in patients who underwent VATS segmentectomy. Taken together, Ki-67 LI may be a predictor of recurrence due to lymphatic involvement even if lymph node metastases were negative.

The selection of segmentectomy over lobectomy during surgery is quite difficult. Therefore, we investigated the possibility of stratifying patients by Ki-67 mRNA expression during the operation. Real-time quantitative RT-PCR is a good tool to evaluate expression levels and one-step PCR is a time-saving method during the operation.<sup>18)</sup> In the present study, we failed to demonstrate that Ki-67 mRNA expression predicted recurrence because this was a low power study. The concordance rates were 73% between mRNA expression by RT-PCR and protein expression by IHC. Underlying post-transcriptional modification may have led to this discrepancy. Further investigation using a large number of patients will be needed to clarify this possibility.

The VATS procedure for lung cancer has increased in the past several years and minimal invasiveness has been shown in previous reports.<sup>19)</sup> The Cancer and Leukemia Group B (CALGB) 140503, phase III trial is ongoing to compare the disease-free survival of patients with small tumors < 2 cm in NSCLC undergoing lobectomy versus sublobar resection. We reported no difference in survival and local recurrence between VATS segmentectomy and VATS lobectomy in stage I NSCLC.<sup>20)</sup> As the selection criteria remain controversial, appropriate patient selection should be developed. The limitations of this study are the relatively short period of follow-up to observe differences in disease-free survival. Longer follow-up and increasing the number of patients in both groups may lead to further confirmation of the results.

In conclusion, although the sample size was small, Ki-67 LI after VATS segmentectomy was a prognostic factor of disease-free survival in NSCLC and the treatment of choice for patients with positive LI may be considered in addition to adjuvant chemotherapy or lobectomy.

### Conflict of interest statement

All authors declare no financial and personal relationships with other people or organizations who might inappropriately influence their work.

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