

Ki-67 labeling index affects tumor infiltration patterns of lung squamous cell carcinoma

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Abstract. Ki-67 is a nuclear protein that is expressed during the G₁, S, G₂ and M phases of the mitotic cell cycle. A previous study categorized tumor infiltration patterns (INF), of which INFc indicated cancer nests exhibiting infiltrative growth and an unclear boundary between tumor tissue and surrounding healthy tissue. The present study used the Ki-67 labeling index (Ki-67 LI) as an indicator of cell proliferation, in order to examine the factors affecting INF in lung squamous cell carcinoma (SqCC). SqCC specimens (89) were classified into two groups: High-grade cell proliferation (Ki-67 LI \geq 30%) and low-grade cell proliferation (Ki-67 LI <30%). However, a high Ki-67 LI was significantly associated with cases that had an INFc component [INFc(+); P=0.03]. Univariate analyses indicated that INFc(+) was a predictor of venous invasion [P=0.032; odds ratio (OR), 2.615; 95% confidence interval (95% CI), 1.085-6.305], scirrhous stromal type (P<0.001; OR, 6.462; 95% CI, 2.483-16.817) and high Ki-67 LI (P=0.018; OR, 12.543; 95% CI, 1.531-102.777). Multivariate logistic analyses indicated that high Ki-67 LI was the strongest predictor of INFc(+) (P=0.028; OR, 8.027; 95% CI, 1.248-51.624). In conclusion, high-grade cell proliferation activity may contribute to aggressive infiltrative growth of lung SqCC.

Introduction

Lung cancer is the most common type of cancer and it is the leading cause of cancer-related mortality worldwide (1). The

prognosis for patients with lung cancer is generally poor, even following complete surgical resection (2), with recurrence rates of 15-30% and 5-year survival rates of 60-70% (3). There is an increasingly broad range of therapeutic options for recurrent or unresectable lung adenocarcinoma, for example, customized chemotherapy (4,5) and molecular-targeted therapies, including bevacizumab (6,7), erlotinib (4,8) and gefitinib (4). By contrast, there are few therapeutic options for recurrent lung SqCC. Therefore, accurate prognostic indicators for patients with lung SqCC are required.

Recent studies have demonstrated immunohistochemical expression patterns of cell-cycle-related molecules in lung cancer, such as p53, retinoblastoma protein, cyclin D1, p27 and Ki-67 (9-13). Ki-67 is a nuclear protein that is expressed during the G₁, S, G₂ and M phases of the mitotic cell cycle, although it is not expressed during the non-mitotic G₀ phase (14-17). The genetic locus of Ki-67 has not yet been characterized. However, it has been assigned to chromosome 10. A number of studies have demonstrated that cell proliferative activity, as indicated by the Ki-67 labeling index (Ki-67 LI), correlates with cell growth (14-17). However, to the best of our knowledge there have been no investigations into the association between Ki-67 LI and lung cancer tumor growth patterns.

Using the general criteria for esophageal and gastric cancer studies (18-25), the tumor infiltration patterns (INF) of lung SqCC have been classified into two groups: Lung tissue specimens with and without clear boundaries between tumor tissue and healthy surrounding tissue, which are termed INFc(-) and INFc(+) respectively (24). Masuda *et al* (24) demonstrated that INFc(+) was significantly associated with venous invasion, the scirrhous stromal tumor type and a lower postoperative survival in patients with lung SqCC. Therefore, INFc(+) may be a useful indicator for the level of local aggressiveness and invasiveness of lung SqCC.

In the present study, the association between INF components and immunohistochemical Ki-67 LI was analyzed. The present study also investigated the clinicopathological significance of cell proliferation and tumor invasiveness at the invasive front of lung SqCCs.

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Materials and methods

Lung cancer specimens. Cancer tissue specimens were obtained from surgically resected lung SqCC tissue following obtaining informed consent from 89 patients (85 males and four females; age range, 43-85 years; mean age, 67.2 ± 0.9 years). The study was approved by the ethics committee of the Institutional Review Board of Tokai University Hospital (Isehara, Japan). All patients had undergone radical surgery (lobectomy and mediastinal lymphadenectomy) at Tokai University Hospital between January 2001 and December 2006. Tumor stages were defined according to the TNM classification of the International Union Against Cancer (UICC; 5), and the histological types were defined according to the World Health Organization classification (26). The median postoperative follow-up duration was 1,572 days (range 41-3,837 days).

Histological examination. Lung tissue specimens were immediately fixed with 10% buffered formalin for 24-48 h and embedded in paraffin (Wako Pure Chemical. Industries Ltd., Osaka, Japan). Tissue samples were cut at 5-10 mm intervals. Tumor invasion and lymphatic invasion were examined in 4- μ m sections, that were stained with hematoxylin and eosin. The extent of lymphatic invasion in the tissue specimens was classified as follows: ly0, no lymphatic invasion; ly1+, mild lymphatic invasion; ly2+, moderate lymphatic invasion; and ly3+, severe lymphatic invasion. Vascular and pleural invasion was evaluated using Elastica van Gieson staining for detection of elastic fibres. The degree of venous invasion in the tissue specimens was classified as follows: v0, no venous invasion; v1+, minimal venous invasion (one or two foci of venous invasion in one histological section); v2+, moderate venous invasion (three or four foci); or v3+, severe venous invasion (five or more foci).

INF at the invasive front of the SqCC was classified into three groups according to the general criteria for gastric cancer studies (18-20,24): INFa, cancer nests exhibit expanding growth and a distinct boundary with the surrounding tissue; INFb, the manner of growth and invasive pattern is intermediate between those of INFa and INFc; and INFc, cancer nests exhibit infiltrative growth without a clear boundary between the tumor tissue and surrounding healthy tissue. However, a number of samples exhibited intermediate characteristics, for example, INFa>b (5,20,27). Therefore, SqCC tissue specimens were further classified into seven categories: INFa, INFa>b, INFa<b, INFb, INFb>c, INFb<c and INFc. These seven categories were allocated into two broader groups: Those cases with an INFc component [INFc(+); comprising INFb>c, INFb<c and INFc], and those without an INFc component [INFc(-); comprising INFa, INFa>b, INFa<b and INFb].

The stromal types, that is, cancer cells/stroma (c/s) ratio in the cancerous lesion were also classified into three groups: Medullary type, the stroma is limited (high c/s ratios); intermediate type, the quantity of stroma is intermediate between those of the scirrhous and medullary types (intermediate c/s ratios); and scirrhous type, the stroma is abundant (low c/s ratios) (19).

Immunohistochemical analysis. Deparaffinized and dehydrated 4- μ m paraffin sections were immersed in

0.3% hydrogen peroxide (H₂O₂) in methanol (Wako Pure Chemical. Industries Ltd.) for 30 min in order to abolish endogenous peroxidase activity. Subsequently, the sections were mounted on aminoacyl silane-coated glass slides and used for immunohistochemical analysis of Ki-67 expression (Ki-67; rabbit monoclonal; cat. no. 418071; Nichirei Bioscience, Tokyo, Japan). In order to facilitate Ki-67 antigen retrieval, the sections were penetrated by autoclave heating (ES-215, High-pressure steam sterilizer; Tomy Seiko Co., LTD, Tokyo, Japan) at 121°C for 4 min. Non-specific binding was abolished using diluted normal sheep serum (Cosmo Bio Co., Ltd, Tokyo, Japan). Subsequently, a primary monoclonal antibody, diluted 1:100 in 1% bovine serum albumin (Wako Pure Chemical. Industries Ltd.) and phosphate-buffered saline (PBS), was added and incubated overnight at 4°C in a moist chamber. Following a wash phase using PBS, a secondary anti-rabbit IgG peroxidase-linked antibody (cat. no. NA934) at 1:100 dilution (Amersham International plc., Little Chalfont, UK) was applied for 60 min at room temperature. The sections were then treated with streptavidin-conjugated horseradish peroxidase for 30 min at room temperature (Funakoshi Co., Ltd., Tokyo, Japan). The reaction products were visualized using diaminobenzidine tetrahydrochloride (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 4 min in Tris buffer.

Evaluation of Ki-67 LI. Cells were observed using a 40x objective microscope (BX50; Olympus, Tokyo, Japan). In each section, $\leq 1,000$ cells were randomly selected and the positive cells were counted. The cut-off point for positivity was considered when $\geq 30\%$ positive cells were observed. Samples were classified into two groups: high-grade cell proliferation (Ki-67 LI $\geq 30\%$) and low-grade cell proliferation (Ki-67 LI $< 30\%$).

Statistical analysis. Univariate analyses (chi-square tests) were primarily used for identifying all variables that exhibited statistically significant differences. $P < 0.05$ was considered to indicate a statistically significant difference. Cox proportional hazards regression analysis was conducted in order to determine the effect of each predictor variable, using univariate analyses. Univariate and multivariate analyses were conducted in order to investigate the association between Ki-67 LI and SqCC tumor invasion. Propensity scores were calculated in the multivariate analysis, in order to measure the effect of the following covariates on INFc(+): Age at surgery, gender, tumor size, lymph node metastasis, lymphatic invasion, histological differentiation and stromal type. Hazard ratios (HR) and 95% confidence intervals (CI) were used to assess the independent contributions of significant factors. In all cases $P < 0.05$ was considered to indicate a statistically significant difference.

The patient survival time was measured from the date of surgery to mortality, related to any cause (without discrimination between mortalities resulting from lung carcinoma and other causes). Survival curves were created using the Kaplan-Meier method and compared using the log-rank test. All analyses were performed using the SPSS II statistical software package (version 19.0; SPSS, Inc., Chicago, IL, USA).

Table I. Ki-67 LI and clinicopathological features of lung squamous cell carcinoma.

Variable	No. patients (%)	Ki-67 LI		P-value
		<30%	≥30%	
Age at surgery (years)				
<68	45 (50.6)	6 (13.3)	39 (86.7)	0.370
≥68	44 (49.4)	9 (20.5)	35 (79.5)	
Gender				
Male	84 (94.4)	12 (14.3)	72 (85.7)	0.032
Female	5 (5.6)	3 (60.0)	2 (40.0)	
Tumor size (mm)				
≤30	34 (38.2)	8 (23.5)	26 (76.5)	0.186
>30	55 (61.8)	7 (12.7)	48 (87.3)	
Lymph node metastasis				
n (-)	62 (69.7)	12 (19.4)	50 (80.6)	0.539
n (+)	27 (30.3)	3 (11.1)	24 (88.9)	
Lymphatic invasion				
ly (0, 1)	75 (84.3)	11 (14.7)	64 (85.3)	0.243
ly (2, 3)	14 (15.7)	4 (28.6)	10 (71.4)	
Venous invasion				
v (-)	47 (52.8)	7 (14.9)	40 (85.1)	0.601
v (+)	42 (47.2)	8 (19.0)	34 (81.0)	
Histological differentiation				
Well, Mod	81 (91.0)	14 (17.3)	67 (82.7)	1.000
Poor	8 (9.0)	1 (12.5)	7 (87.5)	
Stromal type				
Medullary, intermediate	57 (64.0)	11 (19.3)	46 (80.7)	0.411
Scirrhous	32 (36.0)	4 (12.5)	28 (87.5)	
Infiltration pattern				
INFc (-)	55 (61.8)	13 (23.6)	42 (76.4)	0.030
INFc(+)	34 (38.2)	2 (5.9)	32 (94.1)	

INF, tumor infiltration patterns; LI, labeling index; n (-), no lymph node metastasis; n (+), positive lymph node metastasis; ly (0,1), no or mild lymphatic invasion; ly (2,3), moderate or severe lymphatic invasion; v (-), no venous invasion; v (+), positive venous invasion; INFc(-), lung tissue specimen exhibited a clear boundary between tumor tissue and healthy surrounding tissue; INFc(+), cancer nests exhibited infiltrative growth and an unclear boundary between tumor tissue and surrounding healthy tissue.

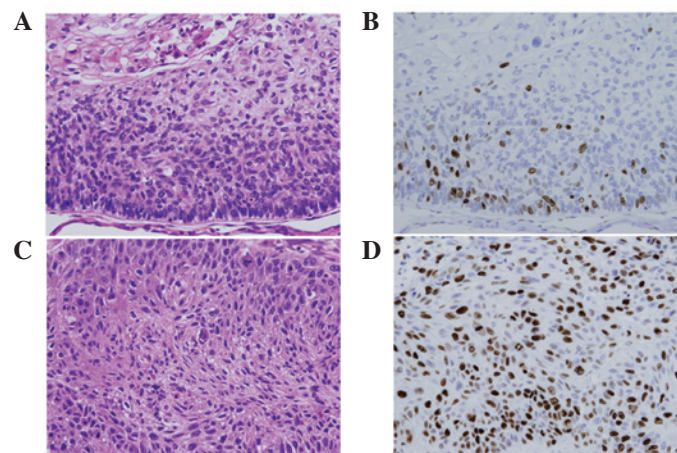


Figure 1. Immunoreactivity of the Ki-67 antibody in the nuclei of lung squamous cell carcinoma cells. (A) Low-grade cell proliferation, hematoxylin and eosin; (B) low-grade cell proliferation, LSAB method; (C) high-grade cell proliferation (≥30% Ki-67 LI), hematoxylin and eosin; (D) high-grade cell proliferation (≥30% Ki-67 LI), LSAB method. X25. LI, labeling index; LSAB, labeled streptavidin-biotin.

Table II. INF in lung squamous cell carcinoma patients.

Variable	Number of patients (%)	Hazard ratio	95% Confidence interval	P-value
INF a	10 (11.2)	0.991	0.392-2.507	0.985
INF a>b, a<b, b, b>c, b<c, c	79 (88.8)			
INF a, a>b	18 (20.2)	1.155	0.556-2.399	0.699
INF a<b, b, b>c, b<c, c	71 (79.8)			
INF a, a>b, a<b	18 (20.2)	1.155	0.556-2.399	0.699
INF b, b>c, b<c, c	71 (79.8)			
INF a, a>b, a<b, b	55 (61.8)	2.069	1.163-3.683	0.013
INF b>c, b<c, c	34 (38.2)			
INF a>b, a<b, b, b>c	82 (92.1)	1.440	0.515-4.027	0.487
INF b<c, c	7 (7.9)			
INF a>b, a<b, b, b>c, b<c	85 (95.5)	1.171	0.283-4.841	0.828
INF c	4 (4.5)			

INF, tumor infiltration patterns; INFa, cancer nests exhibited expanding growth and a clear boundary between tumor tissue and surrounding healthy tissue; INFb, cell growth and invasive patterns were intermediate between those of INFa and INFc; INFc, cancer nests exhibited infiltrative growth and no boundary between tumor tissue and surrounding healthy tissue.

Table III. Association between INFc(+) and clinicopathological factors (univariate analysis).

Variable	No. of patients (%)	Odds ratio	95% Confidence interval	P-value
Age at surgery (years)				
<68	45 (50.6)	1.845	0.776-4.388	0.166
≥68	44 (49.4)			
Gender				
Male	84 (94.4)	1.083	0.172-6.839	0.932
Female	5 (5.6)			
Tumor size (mm)				
≤30	34 (38.2)	0.816	0.340-1.961	0.650
>30	55 (61.8)			
Lymph node metastasis				
n (-)	62 (69.7)	1.166	0.462-2.939	0.745
n (+)	27 (30.3)			
Lymphatic invasion				
ly (0, 1)	75 (84.3)	1.259	0.396-4.005	0.697
ly (2, 3)	14 (15.7)			
Venous invasion				
v (-)	47 (52.8)	2.615	1.085-6.305	0.032
v (+)	42 (47.2)			
Histological differentiation				
Well, Mod	81 (91.0)	0.000	0.000	0.999
Poorly	8 (9.0)			
Stromal type				
Medullary, intermediate	57 (64.0)	6.462	2.483-16.817	<0.001
Scirrhus	32 (36.0)			
Ki-67 LI				
<30%	15 (16.9)	4.952	1.043-23.523	0.044
≥30%	74 (83.1)			

LI, labeling index; n (-), no lymph node metastasis; n (+), positive lymph node metastasis; ly (0,1), no or mild lymphatic invasion; ly (2,3), moderate or severe lymphatic invasion; v (-), no venous invasion; v (+), positive venous invasion; INFc(+), cancer nests exhibited infiltrative growth and an unclear border with the surrounding tissue.

Table IV. Association between INFc(+) and clinicopathological factors (multivariate analysis).

Variable	Odds ratio	95% Confidence interval	P-value
Ki-67 LI			
<30%	12.543	1.531-102.777	0.018
≥30%			
Stromal type			
Medullary, intermediate	8.402	2.923-24.147	<0.001
Scirrhou			
Propensity score	0.025	0.000-1.349	0.070

Ki-67 LI: Ki-67 labeling index; INFc(+), cancer nests exhibited infiltrative growth and an unclear border between the tumor tissue surrounding healthy tissue.

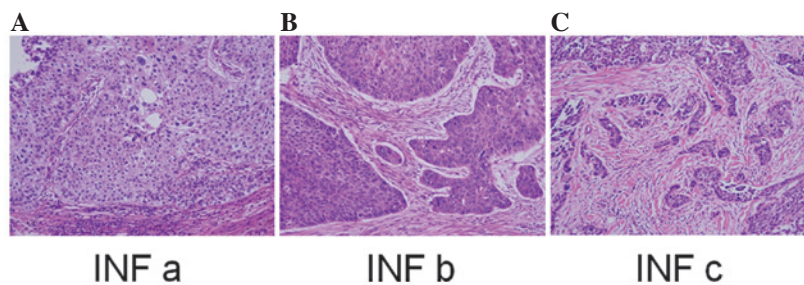


Figure 2. Lung squamous cell carcinoma (hematoxylin and eosin). INF at the invasive front was classified into three groups: (A) INFa, cancer nests exhibited expanding growth and a distinct border between tumor tissue and surrounding healthy tissue. (B) INFb, growth and invasive patterns were intermediate between those of INFa and INFc. (C) INFc, cancer nests exhibited infiltrative growth and an unclear boundary between tumor cells and surrounding healthy tissue. INF, tumor infiltration patterns.

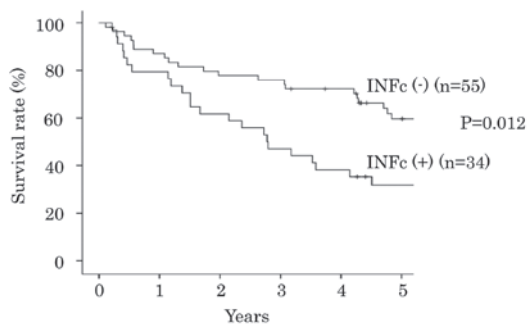


Figure 3. Tumor infiltration patterns and cumulative survival of patients with lung squamous cell carcinoma. INFc, cancer nests exhibited infiltrative growth and an unclear boundary between tumor tissue and surrounding healthy tissue. INF, tumor infiltration patterns.

Results

Lung SqCC cell proliferation. High-grade cell proliferation ($\geq 30\%$ Ki-67 LI) and low-grade cell proliferation ($<30\%$ Ki-67 LI) was observed in 16.9% (15/89) and 83.1% (74/89) of SqCC lung cancer specimens, respectively (Fig. 1). Associations between Ki-67 LI and clinicopathological features are summarized in Table I. INFc(+) was most common in SqCC lung cancer specimens with high-grade cell proliferation ($\geq 30\%$ Ki-67 LI) compared with those with low-grade cell proliferation ($<30\%$ Ki-67 LI; $P=0.03$). However, no

significant difference was detected in prognosis between the high and low Ki-67 LI groups.

Tumor growth patterns of lung SqCC. Lung tissue specimens were initially categorized into three INF groups (Fig. 2). They were further classified into seven groups based on the INF of each specimen: INFa (10, 11.2%), INFa>b (8, 9.0%), INFa<b (0, 0%), INFb (37, 41.6%), INFb>c (27, 30.3%), INFb<c (3, 3.4%) and INFc (4, 4.5%). The patients with INFc(+) (INFb>c, b<c, c) had a poor outcome, compared with the patients with INFc(-) (INFa, a>b, a<b, b) (Table II). The associations between INFc(+) and clinicopathological features of patients with lung SqCC according to a univariate analysis, are summarized in Table III. INFc(+) was significantly associated with venous invasion ($P=0.032$; HR, 2.615; 95% CI, 1.085-6.305), stromal type ($P<0.001$; HR, 6.462; 95% CI, 2.483-16.817) and Ki-67 LI ($P=0.044$; HR, 4.952; 95% CI, 1.043-23.523) of lung SqCC. INFc(+) cases exhibited a significantly poorer prognosis compared with INFc(-) cases ($P=0.012$; Fig. 3).

Multivariate analyses for prediction of INFc. Propensity scores were calculated by measuring the effect of the following covariates on INFc(+): Age at surgery, gender, tumor size, lymph node metastasis, lymphatic invasion, histological differentiation and stromal type. Multivariate logistic regression analysis demonstrated that INFc(+) was significantly associated with Ki-67 LI (odds ratio, 12.5; 95% CI, 1.5-102.8; $P=0.018$) and

stromal type (odds ratio, 8.4; 95% CI, 2.9-24.1; $P < 0.001$) following adjustment for the propensity score (Table IV).

Discussion

As a result of the advances in imaging, diagnostic techniques and operative procedures, the number of patients with lung SqCC undergoing surgical resection has increased for the last three decades. In the present study, 89 surgically resected specimens of lung tissue from patients with lung SqCC were analyzed in order to investigate tumor aggressiveness. INF and local tumor proliferation in lung SqCC were analyzed by measuring cell proliferation, using Ki-67 expression as a proxy. INFc(+) was most common in the lung tissue samples from cases with high-grade cell proliferation (Ki-67 LI $\geq 30\%$) compared with those from cases with low grade cell proliferation (Ki-67 LI $< 30\%$). To the best of our knowledge, this is the first report of an association between INF and lung SqCC cell proliferation.

A previous study demonstrated a correlation between the survival rate of patients with lung SqCC, and tumor budding and histological aggressiveness (24). In the present study, a significantly greater number of INFc(+) lung SqCC specimens exhibited high-grade than low-grade cell proliferation. These results suggest that high-grade cell proliferation may affect the infiltrative growth of cancer nests. Furthermore, INFc(+) was significantly associated with the scirrhous stromal type and positive venous invasion (Table III).

A number of meta-analyses have addressed the prognostic value of Ki-67 in lung cancer. However its clinicopathological role remains to be elucidated (28-34). Ciancio *et al* (9) demonstrated that Ki-67 immunostaining of lung tissue specimens obtained from patients with non-small cell lung cancer (NSCLC) using fiber-optic bronchoscopy, may be useful for making prognostic predictions for patients with lung SqCC. Ciancio *et al* (9) demonstrated that 42.1% of the lung cancer cases exhibited high-grade cell proliferation (Ki-67 LI $> 25\%$). By contrast, the present study demonstrated that 83.1% of SqCC lung cancer specimens exhibited high-grade cell proliferation (Ki-67 LI $> 30\%$). The present study analyzed lung cancer tissues taken from surgical resection, and therefore examined entire tumor, whereas the investigation of Ciancio *et al* (9) used lung cancer specimens obtained by biopsy. It is hypothesized that the different procedure used for obtaining the specimens (biopsy vs. surgical resection) between Ciancio *et al* (9) and the present study, may explain the contrasting results in the percentage of lung cancer specimens exhibiting high-grade cell proliferation. Furthermore, Ciancio *et al* (9) examined Ki-67 overexpression in NSCLC cells and the clinical outcomes for patients with NSCLC, which included SqCC, adenocarcinoma and other histological types. By contrast, the present study focussed on SqCC lung tissue. In terms of patient survival, the results of the present study are in accordance with the conclusions of Ciancio *et al* (9). The present study demonstrated that INFc(+) may be a prognostic factor in SqCC. However, further investigations are required in order to examine the molecular and histological associations between tumor invasiveness and high-grade cell proliferation in lung SqCC.

In conclusion, high-grade cell proliferation, as measured by Ki-67 LI, significantly correlated with an INF that indi-

cated a more aggressive lung SqCC. Ki-67 LI may therefore be used as an indicator of INFc(+) and is a potential prognostic factor for lung SqCC.

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