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ORIGINAL ARTICLE – CANCER RESEARCH

Growth patterns of small peripheral squamous cell carcinoma of the lung and their impacts on pathological and biological characteristics of tumor cells

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Abstract

Purpose The growth pattern of peripheral squamous cell carcinoma (SCC) of the lung is divided into two types: alveolar space-filling (ASF) growth and alveolar space-destructive (ASD) growth. The aim of this study was to investigate the clinicopathological differences between cancer cells displaying ASF and ASD growth.

Methods We analyzed 155 patients with peripheral SCC measuring 30 mm or less in diameter. The proportion of ASF in the total tumor area (%ASF) was determined using digital image analysis. We examined the clinicopathological characteristics of the cancer cells and compared the immunophenotypes of high %ASF tumors (> 30%) and low %ASF tumors (0%). Finally, we analyzed the prognostic impact of ASD area with small SCC cases (≤ 2.0 cm, $n = 72$).

Results Cases of high %ASF tumors showed significantly lower frequencies of lymphovascular invasion ($p = 0.008$). Immunohistochemical staining revealed that the expression score of laminin-5, invasive-related molecule, in cancer cells was significantly lower in high %ASF cases than in low %ASF cases ($p = 0.001$). Within the same tumor, laminin-5 expression in the ASF area was significantly lower than that in the ASD area ($p = 0.001$). The overall 5-year survival rate of patients with a larger ASD area (> 1.0 cm^2) was significantly lower than that of patients with a smaller ASD area (≤ 1.0 cm^2) ($p = 0.017$).

Conclusions In this study, we clearly showed that cancer cells presenting with ASF represents a "less invasive phenotype" in peripheral SCC.

Keywords Peripheral lung cancer · Squamous cell carcinoma · Alveolar space filling (ASF) · Alveolar space destructive (ASD)

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Abbreviations

ASF	Alveolar space filling
ASD	Alveolar space destructive
CD204(+) TAMs	CD204-positive tumor-associated macrophages
CAFs	Cancer-associated fibroblasts
CAIX	Carbonic anhydrase IX
EMT	Epithelial–mesenchymal transition
GLUT-1	Glucose transporter 1
LV	Lymphovascular
PL	Pleural involvement
PD-L1	Programmed death-ligand 1
PDPN	Podoplanin
SCC	Squamous cell carcinoma
SPSS	Statistical Package for Social Science
VVG	Victoria blue van Gieson

HR	Hazard ratio
95% CI	95% Confidence interval

Introduction

Squamous cell carcinoma (SCC) of the lung is the second most common histological type of lung cancer and accounts for approximately 30% of all lung cancers (Perez-Moreno et al. 2012; Tomashefski et al. 1990; Huhti et al. 1983; Parkin et al. 2005; Udagawa et al. 2015; Krimsky et al. 2016). Based on the location of the primary site, SCC can be classified into central and peripheral types (Saijo et al. 2006; Sakurai et al. 2004; Maeshima et al. 2006; Hayashi et al. 2013). Central type lung SCC is generally thought to arise from the bronchial dysplastic epithelium through the dysplasia–carcinoma sequence, a well-known carcinogenic process similar to that observed in uterine cervical neoplasia (Watanabe et al. 2011; Saijo et al. 2006; Carter 1985). However, the early carcinogenic processes of peripheral SCCs have not yet been elucidated (Watanabe et al. 2011; Funai et al. 2003; Mizushima et al. 2000; Malara et al. 1999).

The tumor growth pattern of peripheral SCC can be divided into two types (Watanabe et al. 2011; Funai et al. 2003): alveolar space-filling (ASF) and alveolar space-destructive (ASD) types. ASF type is characterized by growth, separated by thin preexisting septa, that fills the alveolar space without the destruction of the alveolar septa (Watanabe et al. 2011; Funai et al. 2003; Yousem 2009). In contrast, ASD type has no morphologic findings of alveolar space-filling characteristics; instead, it is accompanied by stromal reactions such as inflammatory cell infiltration and fibroblast recruitment. Therefore, there might be biological differences in cancer cells displaying ASF growth and ASD growth.

A number of studies have analyzed the clinicopathological characteristics of ASF type SCC. Funai et al. reported that the 5-year survival rate of patients ($n=5$) showing ASF ratio 100% of the peripheral SCC was 100% (Funai et al. 2003). In addition, Watanabe et al. demonstrated that a high ASF ratio is a significantly favorable prognostic factor in patients with small peripheral SCC of the lung 30 mm or less. They indicated that patients with an ASF ratio over 70% have 100% 5-year survival, suggesting that an ASF ratio of 70% or more in peripheral SCC might be classified as "a microinvasive carcinoma" (Watanabe et al. 2011). These results suggest that cancer cells in an ASF area may have "less aggressive phenotype" than cells in an ASD area in peripheral SCC. However, the biological differences between cancer cells showing ASF and ASD growth have not been clarified.

According to past studies, it was suggested that cancer cells of the ASF area seemingly display "less aggressive phenotype". To this end, we examined two groups by immunohistochemistry.

Materials and methods

Patients

We studied 155 cases of SCC of the lung that were surgically resected at the National Cancer Center Hospital East between February 2004 and December 2013. The study was performed on peripheral SCC of the lung measuring 30 mm or less in diameter. We defined peripheral SCC in this study as tumors located in the third branching bronchus or more peripherally. All cases were classified according to the seventh edition of the International Union Against Cancer TNM classification (Tsim et al. 2010; Wohlschlager et al. 2010). The clinical data of the patients were obtained from medical records. All surgical specimens were collected and analyzed after receiving approval from the Institutional Review Board of the National Cancer Center Hospital East (IRB number 2017-158).

Moreover, we selected patients of SCC measuring 20 mm or less in diameter in this cohort because we hypothesized that to examine the survival difference, smaller tumor would be better. So, we investigated survival analysis of the tumor size with 20 mm or less.

Methods of this study

To clarify the biological differences between cancer cells showing ASF and ASD, we first calculated the proportion of ASF and ASD areas in tumors using digital image analysis and compared the clinicopathological differences between high %ASF tumors and low %ASF tumors. Subsequently, we investigated possible immunophenotypical differences between the two groups.

Pathological studies

Surgical specimens were fixed with 10% formalin and embedded in paraffin. The tumors were cut at approximately 5-mm intervals, and serial 3-μm sections were stained with hematoxylin and eosin and Victoria blue van Gieson (VVG). The histologic diagnoses were based on the fourth edition of World Health Organization histologic classification. In this study, we defined ASF and ASD growth patterns according to the following criteria:

1. ASF growth pattern: tumor cells show growth by filling the alveolar space separated by thin preexisting septa. Desmoplastic stroma are not found within this component (Fig. 1a, c, e).

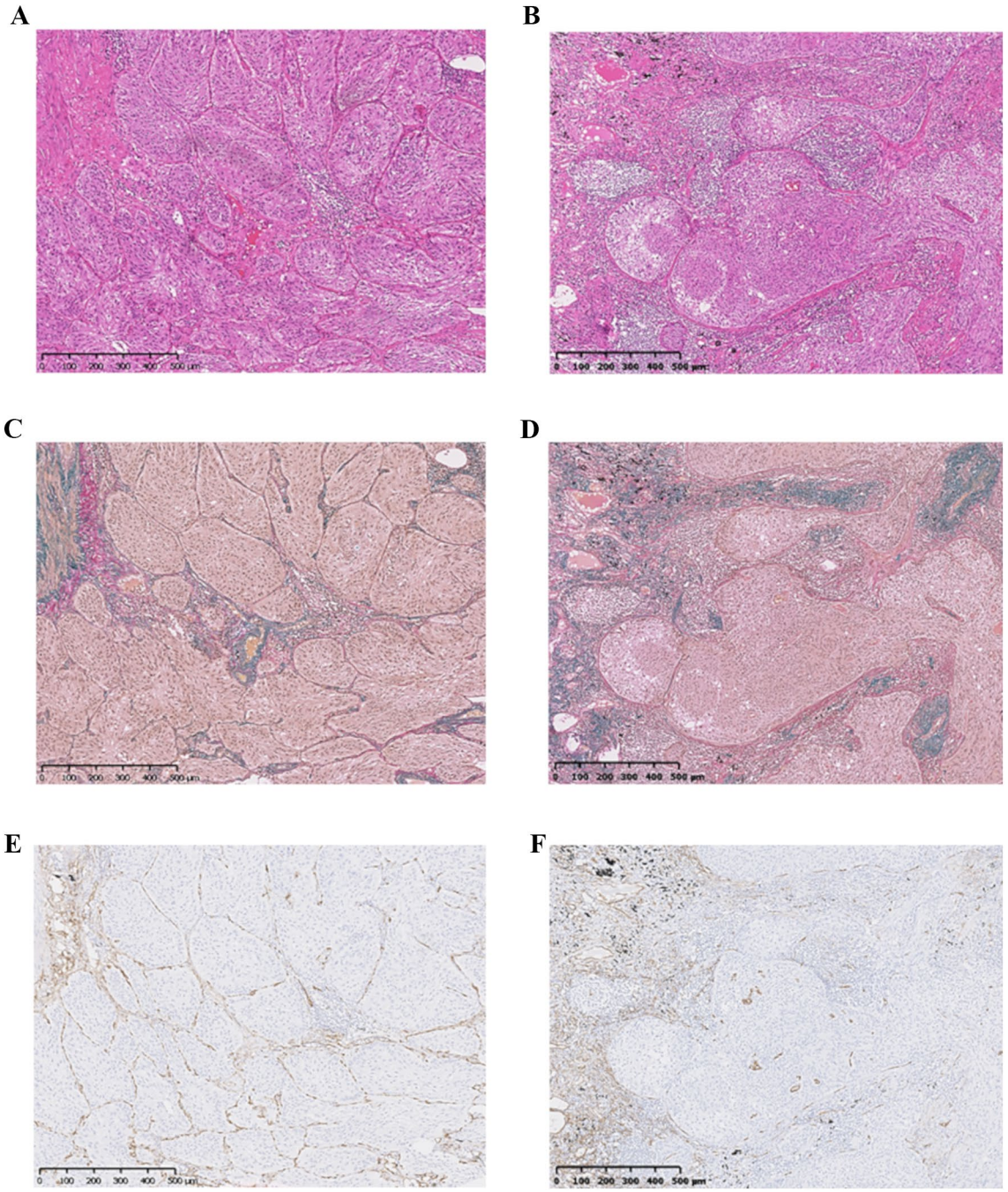

Fig. 1 Histology of alveolar space-filling (ASF) area and alveolar space-destructive (ASD) area of peripheral SCC of the lung. ASF growth pattern: tumor cells show growth by filling the alveolar space separated by thin preexisting septa without stromal reaction ASD growth pattern: tumor cells show solid growth with no morphologic findings of alveolar space-filling characteristics. Histology of ASF area: **a** Staining with hematoxylin and eosin. **c** Staining with victoria blue van Gieson (VVG). **e** Immunohistochemical staining of CD34 Ab. Histology of ASD area: **b** Staining with hematoxylin and eosin. **d** Staining with VVG. **f** Immunohistochemical staining of CD34 Ab

2. ASD growth pattern: tumor cells show solid growth with no morphologic findings of alveolar space-filling characteristics. The tumor cells form irregular-shaped nests, intermingled with an extensive stroma (Fig. 1b, d, f).

Evaluation of ASF and ASD tumor components

To evaluate ASF and ASD tumor components, we stained all sections with VVG stain to observe the elastic

framework of the alveolar septa (Fig. 1c, d). Additionally, we stained CD34 Ab for recognizing capillary vessels of the alveolar septa more clearly (Fig. 1e, f). Area of tumor growth was defined as ASF type when 75% of the total circumference of the tumor nests were surrounded by alveolar septa. Other areas were defined as ASD type.

Calculation of ASF and ASD area

To measure the total tumor, ASF and ASD areas, we first viewed the tumor under the microscope and then outlined the entire tumor circumference and ASF area using blue and red pen, respectively (Fig. 2). In this study, we measured the ASF area first, and the other was defined the ASD area.

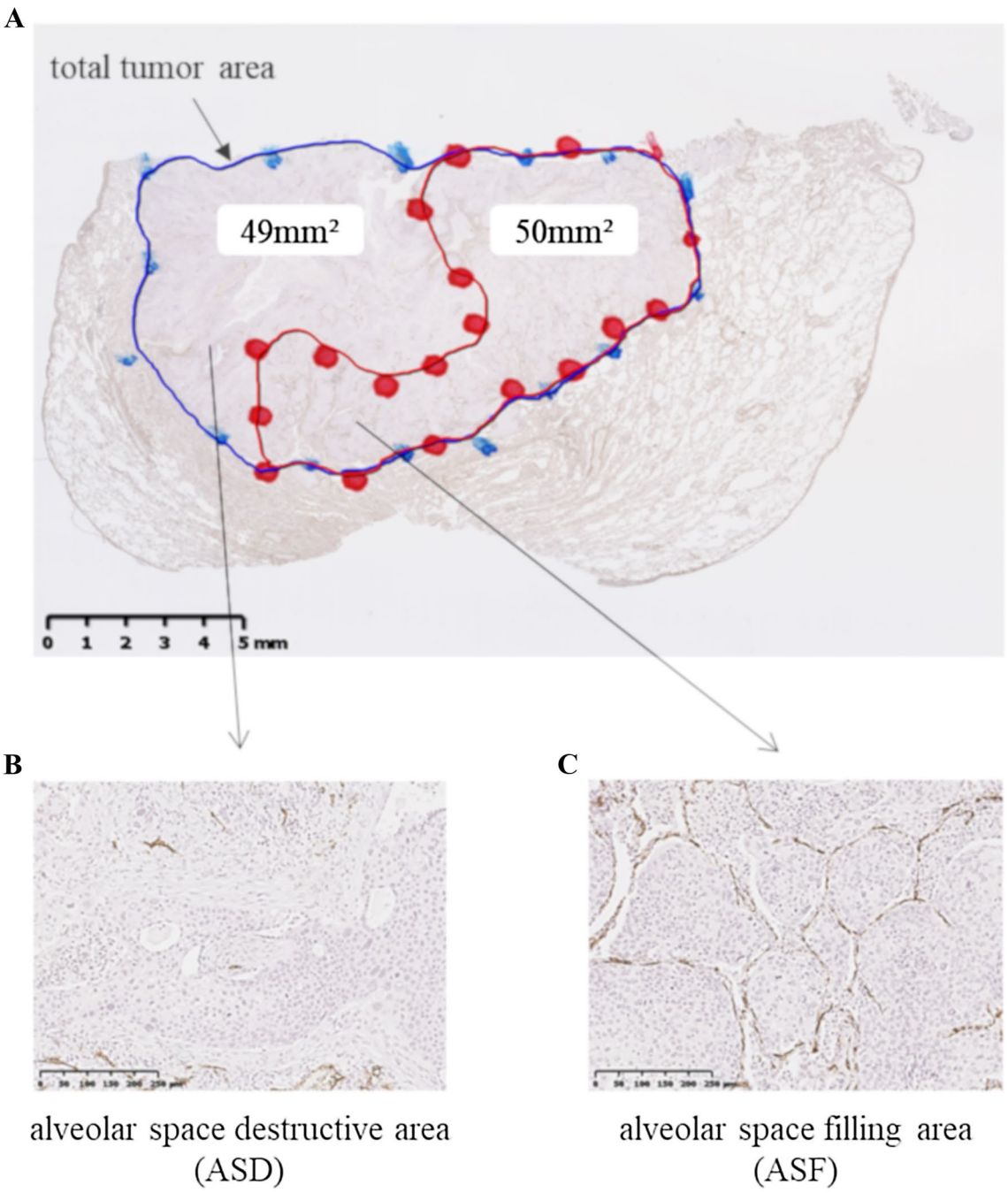

Fig. 2 Digital slide image of ASF and ASD area. Section was stained with CD34 Ab. **a** Tumor area size. Blue line: total tumor area including cancer cells with both ASD and ASF growth pattern. Red line: ASF area including only cancer cells with ASF growth pattern. **b** Higher magnification of ASD area. **c** Higher magnification of ASF area

These two components always add up to 100%. The ASF and ASD areas were determined by two pathologists, and each of these was calculated using a computer with automatic calculation. Subsequently, we captured all slides using NanoZoomer (Hamamatsu Photonics, Japan) slide scanner and measured the size of each area with the NanoZoomer Digital Pathology virtual slide viewer (Hamamatsu Photonics, Japan).

Antibodies and immunohistochemistry

Sections were cut from the paraffin blocks and mounted on silanized slides. Information of primary antibodies used in this study is summarized in Supplementary Table 1. Antigen retrieval was performed in citric buffer solution (pH 6.0). All slides were heated to 95 °C by exposure to microwave irradiation for 20 min. Then, they were allowed to cool for 1 h at room temperature and washed in PBS. As for laminin V, the slides were pretreated with proteinase K (Dako, Glostrup, Denmark) for 10 min. Individual slides were then incubated overnight at 4 °C with different primary antibodies. After extensive washing with phosphate-buffered saline, the slides were incubated with EnVision (Dako) for 1 h at room temperature. The color reaction was developed for 3 min in 2% 3,3 9 -diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxide. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated, and mounted.

Evaluation of immunohistochemistry

Immunostaining scores were evaluated based on the staining intensity and the percentage of cancer cells. Labeling scores were calculated by multiplying the percentage of positive cancer cells per lesion (0–100%) by the staining intensity (0 = negative, 1 = weak, and 2 = strong). Scores ranged between 0 and 200.

The numbers of CD204-positive tumor-associated macrophages (CD204(+) TAMs) were counted under a light microscope using high-power magnification (400×0.0625 mm^2/field). The average count of the three areas was recorded. The staining score of podoplanin-positive cancer-associated fibroblasts (PDPN(+) CAFs) was calculated by multiplying the percentages of PDPN(+) CAFs per tumor area (0–100%) by the immunohistochemical staining intensity (Saruwatari et al. 2016). All immunohistochemically stained slides were evaluated by two pathologists (T.O. and G.I.) who were unaware of the patients' clinical or pathological data. Scoring for the IHC staining was performed independently by the two pathologists. Moreover, we performed face-to-face re-scoring when the scores between the two pathologists were different. The individual sections were evaluated by light microscopy.

We selected the seven molecules (Supplementary Table 1). It is widely known that laminin 5 expression in cancer cells was correlated with the invasion ability (Moriya et al. 2001). In the current study, we would like to examine the invasion ability of tumor cells. We selected other molecules which were reportedly associated with malignant potential.

Statistical analysis

Statistical analysis was performed using the χ^2 test. We established cutoff points of %ASF by ROC statistics. The median follow-up period was 1762 days. Recurrence-free survival was calculated using the Kaplan–Meier method. The length of survival was defined using the log-rank test to select significant factors to discriminate patients who had a relapse from those who were relapse free. To identify which factors had a significant influence on the patients' survival outcome, Cox proportional hazards models were used. As for immunostaining scores, statistical difference was assessed by the Wilcoxon signed-rank test. Statistical significance was set at $p < 0.05$. Statistical Package for Social Science (SPSS) (version 24; IBM Inc.; Chicago, IL, USA) was used to perform all statistical analyses.

Results

Clinicopathological characteristics of the proportion of ASF component

The mean age of patients at the time of surgery was 71 years (range 46–87 years) and the median follow-up time was 4.5 years. Supplementary Fig. 1 shows the number of patients in each ASF ratio group (the proportion of ASF growth in the total tumor area; %ASF). The highest number of patients was found to have a %ASF of 1–10%. We performed ROC curve analysis to determine the cutoff value for lymphovascular (LV) invasion. Sensitivity was 27.1% and specificity was 80.2% and the cutoff point was 23.2. Then we set the cutoff value of %ASF as 30%. When we examined cases containing tumors with ASF ≤ 30% and ASF > 30%, ASF ≤ 30% showed a significantly higher frequency of lymphovascular (LV) invasion ($p = 0.008$) (Table 1).

Immunohistochemical staining results

We hypothesized that the biological characteristics of cancer cells in the ASF component would be different from those in the ASD component. We selected the same condition cases with ASF = 0% and ASF > 30% by the propensity score matching ($n = 27$, each) (Table 2 and Supplementary Table 2) and examined the immunophenotypes of the cancer

Table 1 Clinicopathological characteristics of peripheral squamous cell carcinoma ($n=155$)

Variables	ASF ≤ 30% No. (%)	ASF > 30% No. (%)	*p*
Overall	128	27	
Gender			
Male	111 (86.7)	25 (92.6)	0.398
Female	17 (13.3)	2 (7.4)	
Age (year)			
Median	72	69	0.039
Range	46–87	54–85	
Smoking habit			
Non-smoker	2 (1.5)	0 (0)	0.456
Past smoker	65 (50.8)	12 (44.4)	
Current smoker	61 (47.7)	15 (55.6)	
Tumor size			
≤ 1.0 cm	3 (2.3)	1 (3.7)	0.297
1.0–2.0 cm	54 (42.2)	14 (51.9)	
2.0 cm <	71 (55.5)	12 (44.4)	
Surgical resection			
Wedge	22 (17.2)	3 (11.1)	0.580
Lobar	104 (81.3)	24 (88.9)	
Others	2 (1.5)	0 (0)	
N classification			
N0	114 (89.1)	27 (100)	0.072
N1	4 (3.1)	0 (0)	
N2	10 (7.8)	0 (0)	
N3	0 (0)	0 (0)	
Pleural invasion			
Negative	103 (80.5)	26 (96.3)	0.045
Positive	25 (19.5)	1 (3.7)	
LV invasion			
Negative	69 (53.9)	22 (81.5)	0.008
Positive	59 (46.1)	5 (18.5)	

LV lymphovascular

cells in each group. We also examined the invasive-related molecule, laminin-5; hypoxia-related molecules, GLUT-1 (glucose transporter 1) and CAIX (carbonic anhydrase IX); EMT (epithelial–mesenchymal transition)-related molecule, E-cadherin; stem cell-related molecule, CD44; and immune checkpoint-related molecule, programmed death-ligand 1 (PD-L1) in cancer cells. Moreover, we evaluated the number of tumor-promoting stromal cells, including PDPN(+) CAFs and CD204(+) TAMs. The immunohistochemical staining scores for each molecule in ASF = 0% and ASF > 30% groups are presented in Table 2. Figure 3a, b shows the laminin-5 expression of ASF > 30%, and Fig. 3c, d shows the laminin-5 expression of ASF = 0%. The average scores of laminin-5 were 34.4 and 13.7 in the ASF = 0% and ASF > 30% groups, respectively, and the difference was

Table 2 Immunohistochemical staining score of cancer cells and stromal cells

Variables	ASF component (%)		*p*
	ASF = 0% ($n=27$)	ASF > 30% ($n=27$)	
Cancer cells			
Invasion related			
Laminin-5	34.4	13.7	0.001
Hypoxia related			
GLUT-1	56.7	53.3	0.592
CAIX	13	12.6	0.403
EMT related			
E-cadherin	37.4	55.2	0.056
Stem cell related			
CD44	45.6	46.3	0.944
Podoplanin	10	9.6	0.919
Immune checkpoint related			
PD-L1	15.9	8.1	0.482
Stromal cells			
Podoplanin (+) CAFs	9	10	0.776
CD204 (+) TAMs	23.6	19.1	0.105

significant ($p=0.001$) (Table 2). On the contrary, there were no significant differences between the staining scores of the other molecules (Table 2). E-cadherin expression score of cancer cells in ASF = 0% tumors tended to be lower than that in ASF > 30% ($p=0.056$; Table 2).

As for the tumor-promoting stromal cells, the number of PDPN(+) CAFs and CD204(+) TAMs was not significantly different between the ASF = 0% and ASF > 30% groups (Table 2).

Comparison of laminin-5 expression level in ASF and ASD components within the same tumor

We evaluated whether laminin-5 expression of the ASF area was also lower than that of the ASD area within the same tumor. The cohort used was the ASF > 30% group ($n=27$). Figure 4a–c shows the results of the staining score of laminin-5 in the ASF (Fig. 4b) and ASD areas (Fig. 4c) within the same tumor. The laminin-5 expression score of cancer cells in the ASF area (median 5, range 0–18) was significantly lower than that in the ASD area (median 10, range 0–50) within the same tumor (Wilcoxon signed-rank test, $p=0.001$, Fig. 4d).

Prognostic impact of the ASD area in small peripheral SCC

We measured the actual area of ASD growth in each case of small SCC (≤ 2 cm) and divided the cases into two

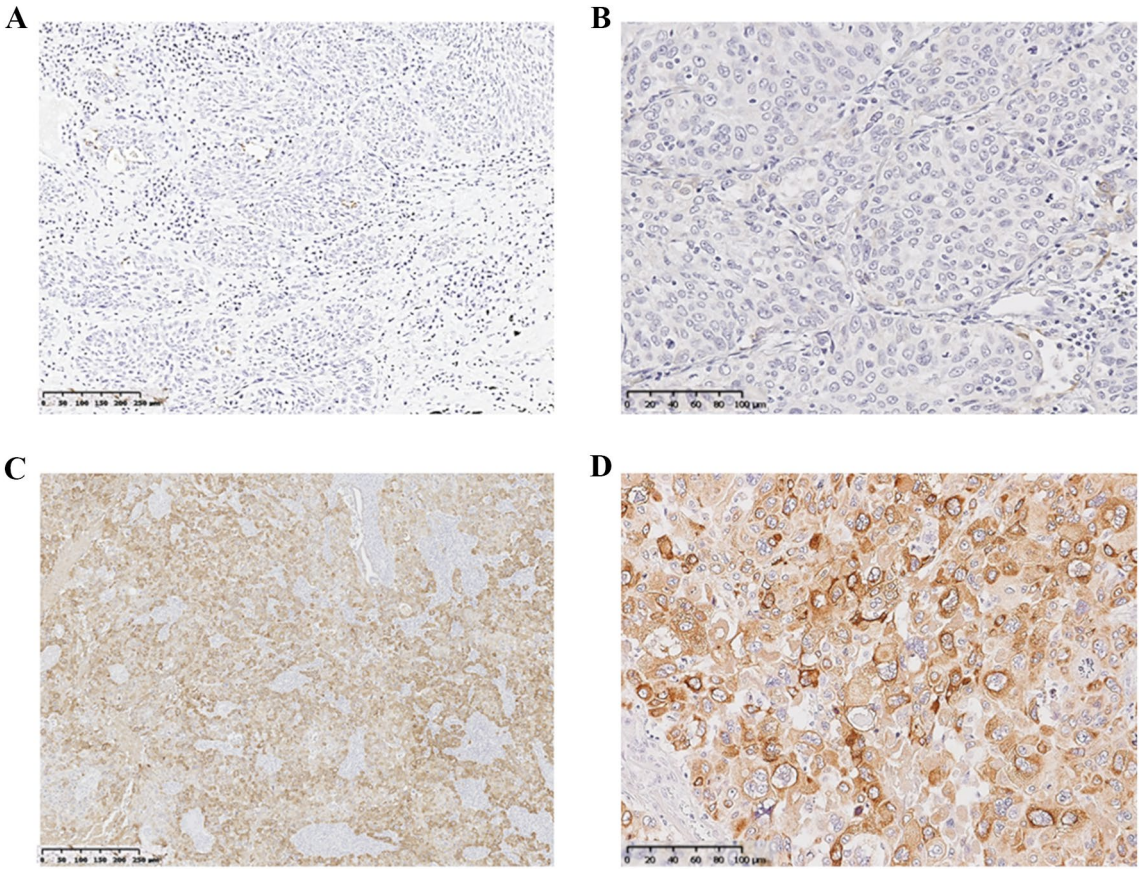

Fig. 3 Immunohistochemical staining of laminin-5 in cancer cells. **a**, **b** Laminin-5 expression in the cytoplasm of cancer cells of ASF > 30% tumor. **c**, **d** Laminin-5 expression in the cytoplasm of cancer cells of ASF = 0% tumor

groups: larger ASD area (> 1.0 cm^2) and smaller ASD area (≤ 1.0 cm^2). The number of cases with a larger ASD area was 24 and that with a smaller ASD area was 48 (Supplementary Table 3). Pleural invasion occurred at a significantly higher rate in cases with a larger ASD area ($p = 0.023$). We performed Cox regression analysis and the result is shown in Supplementary Table 4 and Fig. 5.

On the basis of the multivariate Cox proportional hazards analysis, ASD area [hazard ratio (HR) 5.38, 95% confidence interval (CI) 2.10–13.80, $p < 0.001$], co-morbidity (HR 5.09, 95% CI 1.57–16.53, $p = 0.007$) and surgical resection (HR 0.18, 95% CI 0.07–0.47, $p < 0.001$) were significant independent poor prognostic predictors for OS. Regarding RFS, ASD area (HR 4.02, 95% CI 1.64–9.84, $p = 0.002$), co-morbidity (HR 3.17, 95% CI 1.20–8.38, $p = 0.020$) and surgical resection (HR 0.17, 95% CI 0.07–0.40, $p < 0.001$) were significant independent poor prognostic predictors (Supplementary Table 4). The overall 5-year survival rates in cases with a larger ASD area and a smaller ASD area were 55.7% and 81.7%, respectively. Patients with a smaller ASD area had a significantly higher survival rate than those with a larger ASD area ($p = 0.017$, Fig. 5a). Although not significant, patients with a smaller ASD area tended to have a longer recurrence-free survival than those with a larger ASD area ($p = 0.074$, Fig. 5b).

Discussion

Based on the tumor growth pattern, peripheral SCC of the lung is divided into the following two patterns: ASF type and ASD type (Watanabe et al. 2011; Funai et al. 2003). In previous reports, the presence of the ASF component was recognized as conferring a better survival index; however, few reports have investigated why the presence of larger ASF area could be associated with better survival rate (Watanabe et al. 2011; Funai et al. 2003; Maeshima et al. 2006). In this study, we found that the expression level of the invasive-related marker, laminin-5 (Moriya et al. 2001), in cancer cells within the ASF area was significantly lower than that within the ASD area. In addition, cases with a larger ASD area (> 1.0 cm^2) had a significantly worse survival than those with a smaller ASD area (≤ 1.0 cm^2). These results suggest that cancer cells in the ASD area have a higher invasive

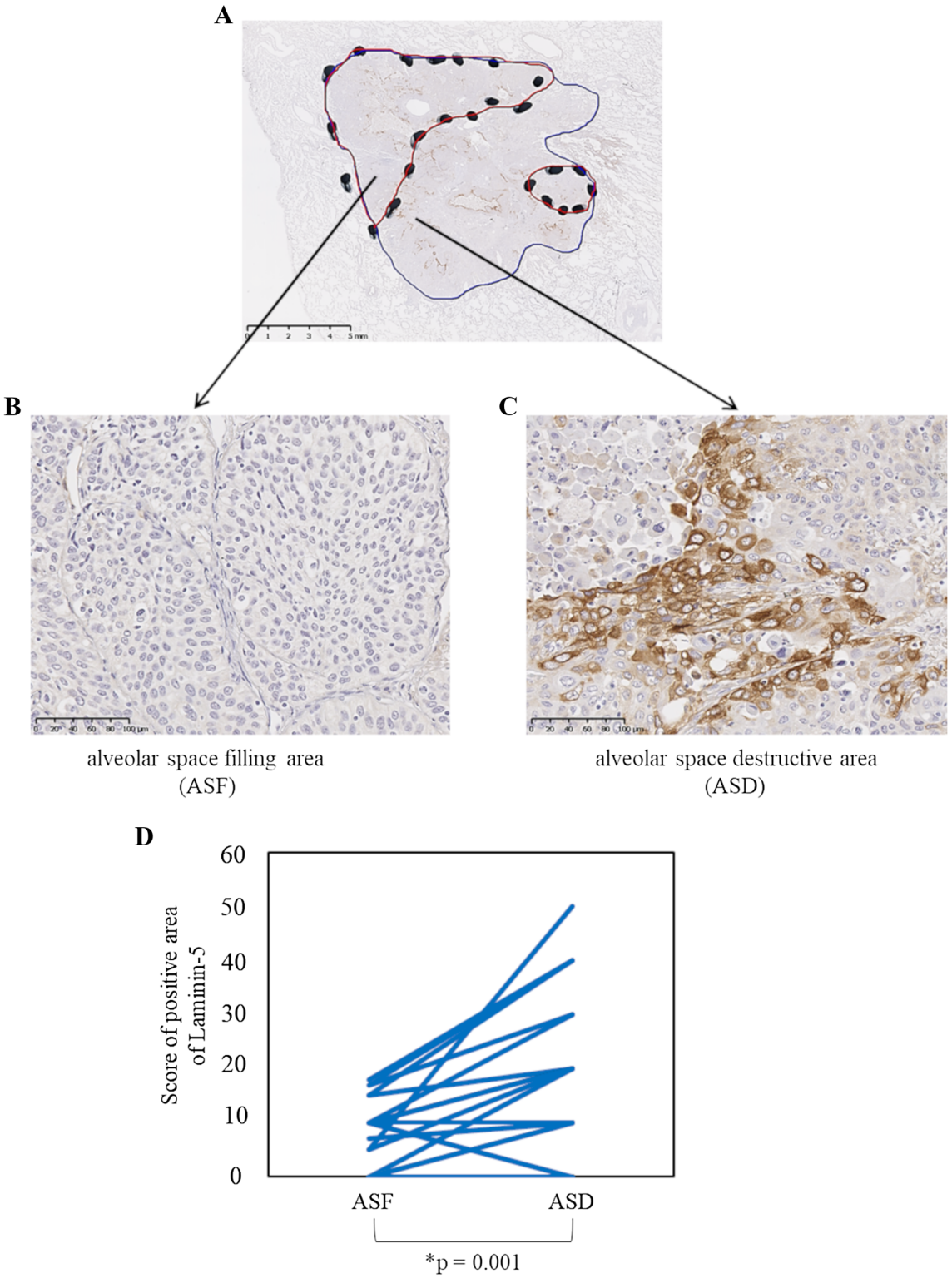

Fig. 4 Immunohistochemical staining result of laminin-5 in ASF and ASD growth within the same tumor. **a** Blue line: total tumor area including cancer cells with both ASD and ASF growth pattern; red line: ASF area including only cancer cells with ASF growth pattern. **b** Laminin-5 expression in ASF area. **c** Laminin-5 expression in ASD area. **d** The expression score of laminin-5 in ASF and ASD areas examined using Wilcoxon signed-rank test

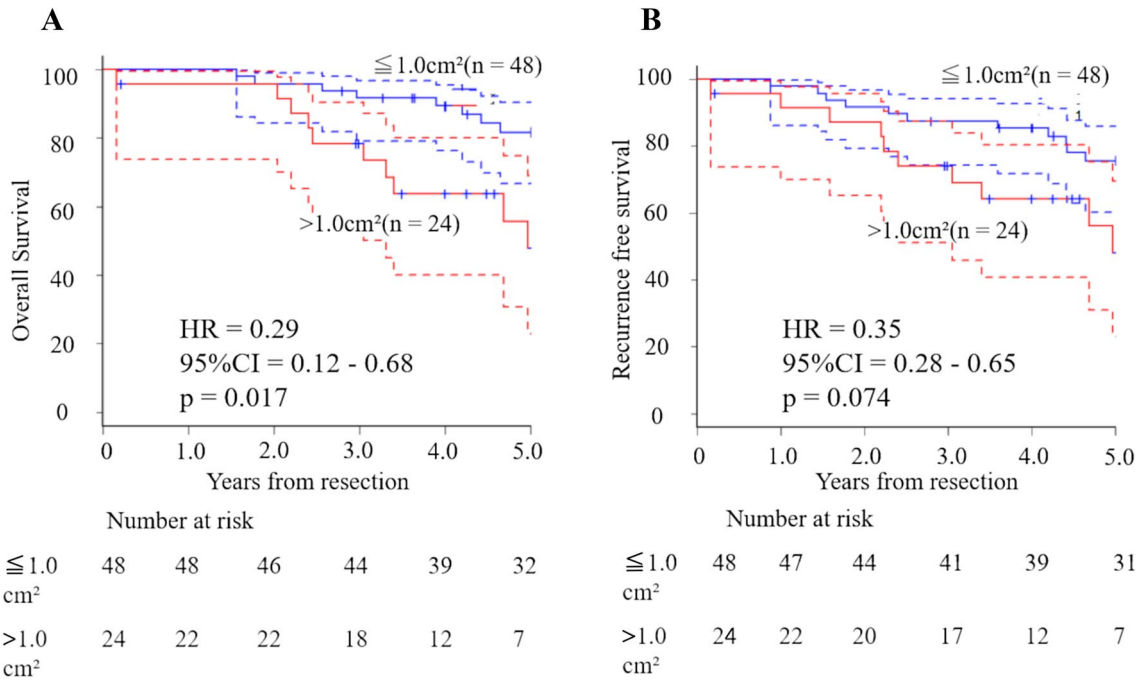

Fig. 5 Kaplan–Meier curve for overall survival and recurrence-free survival in small SCC (≤2 cm) with a smaller ASD area (≤1.0 cm^2) and a larger ASD area (>1.0 cm^2). **a** Overall survival in small SCC (≤2 cm) with a smaller ASD area (≤1.0 cm^2) and a larger ASD area (>1.0 cm^2). **b** Recurrence-free survival in small SCC (≤2 cm) with a smaller ASD area (≤1.0 cm^2) and a larger ASD area (>1.0 cm^2)

potential than those in the ASF area. This is the first report that reveals a biological difference between cancer cells showing ASF and ASD growth patterns in peripheral SCC.

In previous reports, a larger ASF ratio was associated with an improved prognosis (Dingemans and Mooi 1984; Kawabata et al. 1996; Paakko et al. 1990; ten Velde et al. 1991). Therefore, they suggested that the ASF area was "a microinvasive carcinoma", similar to lepidic growth in adenocarcinoma (Travis et al. 2013, 2016; Kadota et al. 2014; Rami-Porta et al. 2015). In this study, when comparing tumors containing ASF ≤ 30% and ASF > 30%, clinicopathological results revealed that the ASF ≤ 30% group showed a significantly higher ratio of local invasion, such as LV invasions ($p = 0.008$). Immunohistochemical staining revealed that the expression score of laminin-5 in cancer cells in the ASF area was significantly lower than that in the ASD area; therefore, cancer cells showing ASF growth would be less invasive. This result was further confirmed by the significantly lower expression score of laminin-5 in the ASF area than in the ASD area within the same tumor.

In small SCCs (≤2 cm), cases with a smaller ASD area had a significantly longer survival than those with a larger ASD area ($p = 0.017$, Fig. 5a), supporting the theory that a smaller ASD area indicates a less invasive area in peripheral small SCC. These results suggest that a smaller ASD area could be a favorable prognostic factor in small peripheral SCC. Further research is required to confirm that ASF or ASD area is an important index of prognostic factor in peripheral SCC with larger size.

Funai et al. reported that 5% (5/109) of patients in their study showed a pure ASF type (Funai et al. 2003). Watanabe et al. reported that the frequency of cases with an ASF ratio 70% or more were 23.5% (Watanabe et al. 2011). However, in our study, there were only two cases (1.3%) with an ASF ratio of 70% or more (Supplementary Fig. 1). We drew an outline of the tumor area for the first time and calculated exactly the tumor and ASF area by using digital image analysis (Fig. 2a), which may be the reason for the discrepancy.

This study had some limitations. Firstly, it is a retrospective study and only one institution was involved. Another limitation of the present study is the relative small number of cases. A larger number of patients should be studied prospectively in the future. In our study, we analyzed the log-rank test by the ASD area (ASD area ≤ 1.0 cm^2 and ASD area > 1.0 cm^2) of all cohort ($n = 155$). Patients with a smaller ASD area tended to have a longer survival than those with a larger ASD area. (OS; $p = 0.08$) Therefore, the result that ASD area was related to prognosis applied only to small squamous cell carcinoma cases.

In conclusion, we revealed that cancer cells with an ASF growth pattern have "less invasive phenotype" in peripheral SCC. Further elucidation of the molecular mechanism by which cancer cells show ASF growth without the destruction

of alveolar septa would be important to strengthen the prognostic significance in peripheral SCC.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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