Podoplanin expression is correlated with the prognosis of lung squamous cell carcinoma

Yoichiro IKOMA¹, Hiroshi KIJIMA³, Ryota MASUDA¹, Makiko TANAKA², Sadaki INOKUCHI², and Masayuki IWAZAKI¹
Departments of ¹General Thoracic Surgery and ²Critical Care and Emergency Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan and ³Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori 036-8562, Japan

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ABSTRACT

Podoplanin is a 38 kDa transmembrane protein that is involved in cell migration and cancer cell invasion. Some studies have reported that podoplanin expression was correlated with poor prognosis in lung squamous cell carcinoma (SqCC). However, there have been no clinicopathological studies of podoplanin membrane expression and localization in lung SqCC. In this study, we focused on the intensity and localization of podoplanin membrane expression, and its clinicopathological significance for lung SqCC. Strong membrane expression of podoplanin was significantly associated with lymph node metastasis, lymphatic invasion, and histological differentiation. Cases with strong podoplanin expression at cell membrane showed better prognosis of lung SqCC (HR, 3.301). Peripheral localization of podoplanin was associated with tumor size, lymphatic invasion, and histological differentiation. Cases with peripheral podoplanin expression showed favorable prognosis of lung SqCC (HR, 2.830). Both strong membrane expression and peripheral expression of podoplanin were independent predictors of mortality of lung SqCC (HR, 2.869; HR, 2.443, respectively). The cases with strong or peripheral podoplanin expression showed better overall survival ($P = 0.001$, both). Podoplanin intensity is significantly associated with podoplanin localization ($P < 0.001$), and its correlation coefficient was 0.678. We concluded that podoplanin membrane expression, not only its localization, is a useful prognostic indicator of lung SqCC patients.

Lung cancer is the leading cause of cancer death globally (2). Despite complete surgical resection, its prognosis is generally poor (1), with recurrence rates of 15–30% and 5-year survival rates of 60–70% (3). Lung cancer is commonly classified into four histological types: squamous cell carcinoma (SqCC), adenocarcinoma, large cell carcinoma, and small cell carcinoma (1, 11), and adenocarcinoma and SqCC are considered as the two major types. Customized chemotherapy for unresectable or recurrent lung

cancers is more frequently used for adenocarcinoma than for SqCC (19, 21). In addition, anti-angiogenic therapy, such as bevacizumab (19, 22), and molecular-targeted therapies, such as erlotinib (5, 20), crizotinib (23, 34), and gefitinib (5), have been developed recently for adenocarcinoma. However, there are limited therapeutic options for unresectable or recurrent SqCC of the lung, and it is therefore important to examine the histopathological features in order to clarify a poor prognosis group of SqCC.

Podoplanin, a 38 kDa transmembrane protein, was identified as an independent platelet aggregation-inducing factor of cancer cells, and is known as a specific marker of lymphatic vessels (8). Recently, podoplanin has been shown to promote epithelial-to-mesenchymal transition (12). Some studies have reported that podoplanin expression was correlated

Address correspondence to: Dr. Hiroshi Kijima, Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan
Tel: +81-17-239-5029, Fax: +81-17-239-5030
E-mail: hkijima@hirosaki-u.ac.jp

with lymph node metastasis, recurrence, and poor prognosis of patients with lung SqCC (6, 15, 18, 27). However, there have been no clinicopathological studies of podoplanin membrane expression and localization in lung SqCC. In this study, we thus focused on the intensity and localization of podoplanin membrane expression, as well as its clinicopathological significance in lung SqCC.

MATERIAL AND METHODS

Patients. One hundred and three lung SqCC cases were surgically resected at Tokai University Hospital from January 2001 to December 2006. All patients provided informed consent, in accordance with the regulations of the Institutional Review Board of Tokai University Hospital. These 103 patients (97 males and 6 females; age range, 43–85 years; mean age, 67.2 ± 9.1 years) with lung SqCC underwent surgery with a radical approach (lobectomy and mediastinum lymphadenectomy) at Tokai University Hospital (Kanagawa, Japan).

Histopathological examination. Lung cancer specimens were routinely fixed with 10% formalin for 24–48 h, embedded in paraffin, cut into 4 μ m sections, and stained with hematoxylin and eosin (H&E). Tumor stages were defined according to the TNM classification of the International Union against Cancer (UICC) (25) and the histological types according to the World Health Organization classification (30). The median postoperative follow-up duration was 1,572 days (range 41–3,837 days). The degree of lymph node metastasis was classified as follows: n0, no lymph node metastasis; n1, ipsilateral peribronchial and/or ipsilateral hilar lymph node metastasis; or n2, ipsilateral mediastinal and/or subcarinal lymph node metastasis, based on the TNM classification (25). The degree of lymphatic invasion was classified as follows: ly0, no lymphatic invasion; ly1, mild lymphatic invasion; ly2, moderate lymphatic invasion; and ly3, severe lymphatic invasion. Then, these categories were divided into two groups: ly0/1 and ly2/3. Vascular and pleural invasion was evaluated using the Verhoeff–van Gieson method. The degree of venous invasion was classified as follows: v0, no venous invasion; v1, minimal venous invasion, that is, one or two foci of venous invasion in one histological section; v2, moderate venous invasion, that is, three or four foci; or v3, severe venous invasion with five or more foci. These were also divided into two groups: v(–) and v(+). Tumor infiltration patterns (INF) at the invasive front were

classified into three groups according to the general rules for gastric cancer studies (4, 7, 11): INFa, cancer nests show expanding growth and a distinct border with the surrounding tissue; INFb, the manner of growth and invasive pattern are intermediate between those of INFa and INFc; and INFc, cancer nests show infiltrative growth and the border with the surrounding tissue is unclear. Some of the cases had two different components of INF, such as INFa > b. Therefore, we divided the cases into seven categories: INFa, INFa > b, INFb > a, INFb, INFb > c, INFb < c, and INFc (13, 14). These seven categories were also divided into two groups: the cases with an INFc component [INFc(+) = INFb > c, INFb < c, and INFc] and those without an INFc component [INFc(–) = INFa, INFa > b, INFb > a, and INFb]. The stromal types, that is, cancer-stroma relationship patterns of lung SqCC, were also classified into three groups: a medullary type with scanty stroma, a scirrhous type with abundant stroma, and an intermediate type, according to the general rules for gastric cancer (10).

Immunohistochemical analysis. Five-micrometer-thick sections were mounted on a silane-coated glass slide and were then deparaffinized and autoclaved (ES-215, high-pressure steam sterilizer; TOMY, Japan) at 121°C for 10 min for antigen retrieval. As primary antibody, an anti-podoplanin antibody (clone D2-40, mouse monoclonal; Nichirei Pharmaceutical, Tokyo, Japan) were prepared for immunohistochemistry. Immunoreactivity was detected using the avidin-biotin method (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA). We evaluated the existence of specific immunostaining prior to the immunohistochemical staining in this study (29).

Evaluation of immunohistochemistry. Immunohistochemically, podoplanin staining intensity was determined at the cancer-stroma interface of lung SqCC, and was classified into three groups as follows: 1) strong, podoplanin was present at the complete cell membrane ($\geq 10\%$ complete membrane staining of podoplanin-positive cells) (Fig. 1A, B); 2) weak, podoplanin was present at the incomplete cell membrane or weakly in the cytoplasm ($\geq 10\%$ incomplete membrane staining of podoplanin-positive cells) (Fig. 1C, D); and 3) negative, podoplanin expression was absent or present at the incomplete membrane expression ($< 10\%$ membrane staining of podoplanin-positive cells). Then, staining localization was determined at the cancer-stroma interface of lung SqCC, and was classified into two groups as follows: 1)

Fig. 1 Immunohistochemical expression of podoplanin (intensity). Squamous cell carcinoma cells invade the stroma (two representative cases: A and C; H&E). Strong: podoplanin is present at the complete cell membrane of lung SqCC (B: the same case as A). Weak: podoplanin is present at the incomplete cell membrane or weakly in the cytoplasm of lung SqCC (D: the same case as C).

Fig. 2 Immunohistochemical expression of podoplanin (localization). Squamous cell carcinoma cells invade the stroma (two representative cases: A and C; H&E). Peripheral type (P type): Immunostaining is observed in the peripheral cells of the tumor nests (B: the same case as A). Non-peripheral type (non-P type): Immunostaining is observed in the random cells of the tumor nests (D: the same case as B).

Table 1 Podoplanin membrane staining and clinicopathological features of lung squamous cell carcinoma

Variable	No. of patients (%)	Podoplanin staining intensity		P-value
		strong	negative/weak	
Age at surgery (years)				
<68	53 (51.5)	17 (32.1)	36 (67.9)	0.820
≥68	50 (48.5)	15 (30.0)	35 (70.0)	
Gender				
Male	97 (94.2)	31 (32.0)	66 (68.0)	0.432
Female	6 (5.8)	1 (16.7)	5 (83.3)	
Tumor size (mm)				
≤30	39 (37.9)	14 (35.9)	25 (64.1)	0.408
>30	64 (62.1)	18 (28.1)	46 (71.9)	
Lymph node metastasis				
n (-)	70 (68.0)	27 (38.6)	43 (61.4)	0.017
n (+)	33 (32.0)	5 (15.2)	28 (84.8)	
Lymphatic invasion				
ly (0, 1)	83 (80.6)	30 (36.1)	53 (63.9)	0.023
ly (2, 3)	20 (19.4)	2 (10.0)	18 (90.0)	
Venous invasion				
v (-)	53 (51.5)	19 (35.8)	34 (64.2)	0.280
v (+)	50 (48.5)	13 (26.0)	37 (74.0)	
Histological differentiation				
well	28 (27.2)	15 (53.6)	13 (46.4)	0.005
mod	62 (60.2)	16 (25.8)	46 (74.2)	
poorly	13 (12.6)	1 (7.7)	12 (92.3)	
Stromal type				
medullary, intermediate	70 (68.0)	20 (28.6)	50 (71.4)	0.425
scirrhous	33 (32.0)	12 (36.4)	21 (63.6)	
Infiltrating pattern				
INFc (-)	64 (62.1)	21 (32.8)	43 (67.2)	0.624
INFc (+)	39 (37.9)	11 (28.2)	28 (71.8)	

n (-)/n (+): lymph node metastasis-negative/positive

ly: lymphatic invasion: (0) negative; (1) mild; (2) moderate; and (3) severe

v (-)/v (+): venous invasion-negative/positive

INFc (-)/INFc (+): INFc component-negative/positive

peripheral, immunostaining was observed in the peripheral cells of the tumor nests (Fig. 2A, B); 2) non-peripheral immunostaining was observed in the random cells of the tumor nests or was not observed in any tumor cells (Fig. 2C, D).

Statistical analysis. Univariate analysis was performed by chi-square test. Cox proportional hazards regression analysis was used to determine the net effects of each predictor while controlling for the effect of the other factors by uni- and multivariate analyses. Independent prognostic factors were analyzed by Cox's proportional hazard regression model. Hazard ratios (HR) and their 95% confidence intervals (CI) were used to assess the independent contributions of significant factors. $P < 0.05$ was considered to indicate a statistically significant dif-

ference. The patients' overall survival was measured from the date of operation; death from all causes (without discrimination between deaths resulting from esophageal squamous cell carcinoma and other causes) was taken as the outcome. Survival curves were calculated by the Kaplan-Meier method and analyzed by the log-rank test. All analyses were performed using the SPSS statistical software, version 21 (SPSS, International Business Machines C.P., Armonk, NY, USA).

RESULTS

Histological expression of podoplanin

Podoplanin was strongly expressed at the lymphatic endothelial cells of non-neoplastic lung tissue as internal controls. Podoplanin membrane expression

Table 2 Clinicopathological features and survival in lung squamous cell carcinoma patients

Variable	No. of patients	P-value	Hazard ratio	95% Confidence interval
Age at surgery (years)				
<68	53 (51.5)	0.131	1.502	0.885–2.548
≥68	50 (48.5)			
Gender				
Male	97 (94.2)	0.904	0.939	0.339–2.602
Female	6 (5.8)			
Tumor size (mm)				
≤30	39 (37.9)	0.031	1.897	1.059–3.396
>30	64 (62.1)			
Lymph node metastasis				
n (–)	70 (68.0)	<0.001	3.028	1.785–5.136
n (+)	33 (32.0)			
Lymphatic invasion				
ly (0, 1)	84 (81.6)	<0.001	3.416	1.909–6.113
ly (2, 3)	19 (18.4)			
Venous invasion				
v (–)	53 (51.5)	0.145	1.486	0.873–2.530
v (+)	50 (48.5)			
Histological differentiation				
well, mod	90 (87.4)	0.036	2.092	1.050–4.168
poorly	13 (12.6)			
Stromal type				
medullary, intermediate	70 (68.0)	0.465	1.229	0.706–2.139
scirrhous	33 (32.0)			
Infiltrating pattern				
INFc (–)	64 (62.1)	0.003	2.209	1.301–3.749
INFc (+)	39 (37.9)			
Podoplanin staining intensity				
strong	32 (31.1)	0.001	3.301	1.610–6.767
negative, weak	71 (68.9)			
Podoplanin localization				
peripheral type	36 (35.0)	0.002	2.830	1.456–5.500
non-peripheral type	67 (65.0)			

n (–)/n (+): lymph node metastasis-negative/positive

v (–)/v (+): venous invasion-negative/positive

ly: lymphatic invasion: (0) negative; (1) mild; (2) moderate; and (3) severe

INFc (–)/INFc (+): INFc component-negative/positive

was detected in 66.0% (68/103) of lung SqCC, that is, weak expression in 35.0% (36/103) and strong expression in 31.1% (32/103). In terms of the localization of podoplanin expression, peripheral type was found in 35.0% (36/103), and non-peripheral type in 65.0% (67/103).

Tumor growth patterns of lung SqCC

In terms of the INF patterns, the 103 lung SqCC cases were classified as follows: INFa (11, 10.7%), INFa > b (10, 9.7%), INFa < b (0, 0%), INFb (43, 41.7%), INFb > c (31, 30.1%), INFb < c (4, 3.9%), and INFc (4, 3.9%). The cases with an INFc com-

ponent [INFc(+) = INFb > c, INFb < c, and INFc] showed a poor patient's outcome compared with those without an INFc component [INFc(–) = INFa, INFa > b, INFb > a, and INFb] (24). The stromal types were divided as follows: medullary type (35, 34.0%), intermediate type (35, 34.0%), and scirrhous type (33, 32.0%).

Clinicopathological findings and the podoplanin intensity of lung SqCC

The relationships between the podoplanin intensity and clinicopathological features are summarized in Table 1. Strong podoplanin expression was signifi-

Table 3 Clinicopathological features and survival in lung squamous cell carcinoma patients

Variable	No. of patients	P-value	Hazard ratio	95% Confidence interval
Podoplanin staining intensity				
strong	32 (31.1)	0.005	2.869	1.377–5.976
negative, weak	71 (68.9)			
Age at surgery (years)				
<68	53 (51.5)	0.048	1.723	1.006–2.951
≥68	50 (48.5)			
Gender				
Male	97 (94.2)	0.465	0.678	0.239–1.922
Female	6 (5.8)			
Tumor size (mm)				
≤30	39 (37.9)	0.241	1.447	0.780–2.682
>30	64 (62.1)			
Lymph node metastasis				
n (–)	70 (68.0)	<0.001	2.753	1.570–4.827
n (+)	33 (32.0)			

n (–)/n (+): lymph node metastasis-negative/positive

cantly correlated with lymph node metastasis ($P = 0.017$), lymphatic invasion ($P = 0.023$), and histological differentiation ($P = 0.005$). The aforementioned univariate analyses identified six factors associated with increased mortality in patients with lung SqCC (Table 2): tumor size (HR, 1.897; 95% CI, 1.059–3.396, $P = 0.031$), lymph node metastasis (HR, 3.028; 95% CI, 1.785–5.136, $P < 0.001$), lymphatic invasion (HR, 3.416; 95% CI, 1.909–6.113, $P < 0.001$), histological differentiation (HR, 2.092; 95% CI, 1.050–4.168, $P = 0.036$), tumor infiltrative patterns (HR, 2.209; 95% CI, 1.301–3.749, $P = 0.003$), and podoplanin staining intensity (HR, 3.491; 95% CI, 1.643–7.416, $P = 0.001$). Multivariate analysis demonstrated that strong podoplanin expression (HR, 2.869; 95% CI, 1.377–5.970, $P = 0.005$) and lymph node metastasis (HR, 2.753; 95% CI, 1.570–4.827, $P < 0.001$) were independent predictors of patients' mortality in lung SqCC (Table 3). Cases of strong podoplanin expression showed significantly better overall survival than negative or weak cases ($P = 0.001$, log-rank test, Fig. 3A).

Clinicopathological findings and the podoplanin localization of lung SqCC

The relationships between podoplanin localization and clinicopathological features are summarized in Table 4. The peripheral type was significantly correlated with tumor size ($P = 0.022$), lymphatic invasion ($P = 0.037$), and histological differentiation ($P = 0.001$). The aforementioned univariate analyses identified that the non-peripheral type was also associated with increased mortality in patients with

Fig. 3 Patient survival and podoplanin expression. Cases of strong podoplanin expression show significantly better overall survival than negative and weak cases ($P = 0.001$, log-rank test) (A). Cases of peripheral podoplanin expression (P type) also show significantly better overall survival than negative or diffuse cases (non-P type) ($P = 0.001$, log-rank test) (B).

Table 4 Podoplanin localization and clinicopathological features of lung squamous cell carcinoma

Variable	No. of patients (%)	P type	Non-P type	P-value
Age at surgery (years)				
<68	53 (51.5)	21 (39.6)	32 (60.4)	0.306
≥68	50 (48.5)	15 (30.0)	35 (70.0)	
Gender				
Male	97 (94.2)	35 (36.1)	62 (63.9)	0.333
Female	6 (5.8)	1 (16.7)	5 (83.3)	
Tumor size (mm)				
≤30	39 (37.9)	19 (48.7)	20 (51.3)	0.022
>30	64 (62.1)	17 (26.6)	47 (73.4)	
Lymph node metastasis				
n (-)	70 (68.0)	28 (40.0)	42 (60.0)	0.118
n (+)	33 (32.0)	8 (24.2)	25 (75.8)	
Lymphatic invasion				
ly (0, 1)	84 (81.6)	33 (39.8)	50 (60.2)	0.037
ly (2, 3)	19 (18.4)	3 (15.0)	17 (85.0)	
Venous invasion				
v (-)	53 (51.5)	23 (43.4)	30 (56.6)	0.064
v (+)	50 (48.5)	13 (26.0)	37 (74.0)	
Histological differentiation				
well	28 (27.2)	17 (60.7)	11 (39.3)	0.001
mod	62 (60.2)	18 (29.0)	44 (71.0)	
poorly	13 (12.6)	1 (7.7)	12 (92.3)	
Stromal type				
medullary, intermediate	70 (68.0)	23 (32.9)	47 (67.1)	0.516
scirrhous	33 (32.0)	13 (39.4)	20 (60.6)	
Infiltrating pattern				
INFc (-)	64 (62.1)	23 (35.9)	41 (64.1)	0.788
INFc (+)	39 (37.9)	13 (33.3)	26 (66.7)	

n (-)/n (+): lymph node metastasis-negative/positive

v (-)/v (+): venous invasion-negative/positive

ly: lymphatic invasion: (0) negative; (1) mild; (2) moderate; and (3) severe

INFc (-)/INFc (+): INFc component-negative/positive

lung SqCC (HR, 2.830; 95% CI, 1.456–5.500, $P = 0.002$) (Table 2). Multivariate analysis demonstrated that podoplanin localization (HR, 2.443; 95% CI, 1.202–4.964, $P = 0.014$) and lymph node metastasis (HR, 3.029; 95% CI, 1.724–5.320, $P < 0.001$) were independent predictors of patients' mortality in lung SqCC (Table 5). Cases of peripheral podoplanin expression showed better overall survival than negative or diffuse cases ($P = 0.001$, log-rank test, Fig. 3B).

Relationships between podoplanin intensity and localization

The relationship between podoplanin intensity and localization is shown in Table 6. Strong podoplanin expression was significantly correlated with peripheral type ($P < 0.001$), with a correlation coefficient of 0.678.

DISCUSSION

In this study, we focused on podoplanin membrane expression and localization at the tumor-stroma interface of lung SqCC, and considered that the membrane expression was more important because the podoplanin molecule is a transmembrane protein. The podoplanin membrane expression was correlated with lymph node metastasis, lymphatic invasion, histological differentiation, and poor prognosis of lung SqCC. Strong podoplanin membrane expression was an independent predictor of the prognosis of patients with lung SqCC. This is the first report to clarify the relationship between podoplanin membrane expression and clinicopathological factors.

Shimada *et al.* reported that positive podoplanin expression was significantly correlated with better prognosis of lung SqCC (24). Using immunohisto-

Table 5 Clinicopathological features and survival in lung squamous cell carcinoma patients

Variable	No. of patients	P-value	Hazard ratio	95% Confidence interval
Podoplanin localization				
P type	36 (35.0)	0.014	2.443	1.202–4.964
non-P type	67 (65.0)			
Age at surgery (years)				
<68	53 (51.5)	0.100	1.585	0.916–2.743
≥68	50 (48.5)			
Gender				
Male	97 (94.2)	0.514	0.707	0.250–1.999
Female	6 (5.8)			
Tumor size (mm)				
≤30	39 (37.9)	0.517	1.237	0.651–2.349
>30	64 (62.1)			
Lymph nodal metastasis				
n (–)	70 (68.0)	<0.001	3.029	1.724–5.320
n (+)	33 (32.0)			

n (–)/n (+): lymph node metastasis-negative/positive

P type: peripheral type

non-P type: non-peripheral type

Table 6 Podoplanin staining intensity and localization of lung squamous cell carcinoma

Variable	No. of patients (%)	Podoplanin staining intensity		P-value
		strong	negative/weak	
Podoplanin localization				
non-P type	36 (35.0)	27 (75.0)	9 (25.0)	<0.001
P type	67 (65.0)	5 (7.5)	62 (92.5)	

Spearman's correlation coefficient = 0.696

P type: peripheral type

non-P type: non-peripheral type

chemical staining of podoplanin, CD44, and P63, they demonstrated the hierarchical distribution within tumor nests, *i.e.* podoplanin-positive cells were more localized to peripheral area than the CD44-positive and p63-positive cells; and p63-positive cells were broader than the CD44-positive cells. Then they showed that patients with the hierarchical distribution had significantly better overall survival. Almost all cases with this pattern are thought to be included in the peripheral type that we have proposed in this study. Suzuki *et al.* mentioned that high podoplanin expression was significantly correlated with a lower incidence of lymph node metastasis in lung SqCC (27). Ito *et al.* reported that the cases with low podoplanin expression showed poor prognosis in lung SqCC (6). However, these reports did not describe the significance of podoplanin membrane expression that we focused on in this study. We demonstrated two different evaluations, namely, intensity and localization, in the present

study. Even if strong or peripheral podoplanin expression is used for evaluation, the cases with strong or peripheral type showed favorable overall survival. The strong type and the peripheral type have a positive linear correlation (correlation coefficient 0.678). The univariate analyses identified both the strong type and the peripheral type as being associated with decreased mortality (HR, 3.301; HR, 2.830, respectively), and each type was identified as a predictor of patients' mortality in lung SqCC. The intensity of podoplanin expression was considered as a useful indicator of lung SqCC.

Recently, podoplanin has been considered as one of the candidate markers of cancer stem cells, associated with cancer cell invasion or migration, as well as the prognosis of several cancers (12, 24, 28, 29, 33). Several studies have clarified that podoplanin expression promotes epithelial-to-mesenchymal transition in morphogenetic processes. Epithelial-to-mesenchymal transition of cancer cells is a phenotypic

change by which epithelial cells lose their polarity, epithelial markers, such as E-cadherin, and obtain migratory factors, such as vimentin and snail which is one of the transcription factors playing an important role in epithelial-to-mesenchymal transition (9, 17, 26, 31, 32). Vimentin expression is one of the good indicators for epithelial-to-mesenchymal transition of carcinoma, which is considered to be associated with aggressive phenotypes with invasive/metastatic potential (16). However, the relationship between vimentin and podoplanin has remained unclear. Therefore, we plan to perform studies to clarify relationship in lung SqCC.

In conclusion, the intensity or localization of podoplanin expression is a useful indicator of prognosis in lung SqCC. In particular, the intensity, that is, membrane expression, of lung SqCC is associated with locally aggressive growth and is a useful indicator of lymph node status and prognosis.

DISCLOSURE

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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