

Expression of laminin-5 γ 2 chain in intrabronchial lesions during carcinogenesis of the bronchial epithelium and in squamous cell carcinoma of the lung

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Abstract

Laminin 5 plays an important role in cell migration during tumor invasion and tissue remodeling. Although this chain-like molecule is reportedly expressed in the tumor-stroma interface of squamous cell carcinoma (SCC) of the lung, the prognostic significance of its expression has not been elucidated. Therefore, we investigated the clinicopathologic significance of laminin-5 γ 2 chain expression in SCC of the lung and the localization of laminin-5 γ 2 chain expression in intrabronchial precancerous lesions. Using immunohistochemistry, we investigated the distribution of the laminin-5 γ 2 chain in 14 cases of intrabronchial precancerous lesions, 1 case of microinvasion to bronchial wall, 21 cases of intrabronchial squamous cell carcinoma and 57 cases of SCC of the lung. Laminin-5 γ 2 was expressed in the cytoplasm of tumor cells. Tumor cells at the cancer-stromal interface and at the invasive front frequently showed immunopositivity for laminin-5 γ 2 in both intrabronchial precancerous lesions and SCC of the lung. Moreover, this protein was strongly expressed in cancer cells showing scattered invasion. Laminin-5 γ 2 expression was correlated significantly with prognosis ($p=0.0151$). These results suggest that expression of laminin-5 γ 2 is a relatively early event in the genesis of bronchial squamous cell carcinoma, and that increased laminin-5 γ 2 immunoreactivity is a significant predictor of poor outcome in patients with SCC of the lung

Introduction

Laminins are a family of glycoproteins found in the extracellular matrix. Their functions include development and maintenance of the basement membrane, and regulation of cell adhesion, migration and differentia-

tion^{1–3)}. Structurally, the basic laminin molecule is a cross-shaped heterotrimer of polypeptide chains, consisting of one heavy chain (α) and two light chains (β and γ)¹⁾²⁾. Differences in these chains and their combinations produce a variety of laminin isoforms that are tissue-specific and probably have different functions³⁾.

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So far, 11 laminin isoforms have been identified⁴⁾. Laminin-5, consisting of the laminin $\alpha 3$, $\beta 3$, and $\gamma 2$ chains, is involved in the attachment of keratinocytes to the basement membrane within the dermal-epidermal junction through interaction with $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, facilitating epithelial-mesenchymal cohesion *in vivo*⁵⁾⁶⁾. In addition, laminin-5 plays a major role in epithelial cell motility, and has been shown to promote vigorous cell scattering when added to the medium of epithelial cell cultures⁷⁾. In particular, laminin-5 $\gamma 2$ is an important molecule for cell motility, and has been detected at the leading margin of invasive colon and breast cancers⁸⁾⁹⁾. Specific cleavage of the laminin-5 $\gamma 2$ chain by matrix metalloproteinase-2 (MMP-2) has been reported to be critical for cell migration during tumor invasion and tissue remodeling¹⁰⁾, and this molecule is reportedly expressed in cancer cells located at the tumor-stroma interface in some human carcinomas⁸⁾¹¹⁾. It has been reported that increased laminin-5 $\gamma 2$ immunoreactivity, which may reflect a high invasive potential of cancer cells, is a factor indicating a poor prognosis for patients with various cancers^{12–15)}. However, previous studies on the relationship between laminin-5 $\gamma 2$ in lung cancer and prognosis have focused only on adenocarcinoma. In this study, we investigated the prognostic significance of laminin-5 $\gamma 2$ chain expression in SCC of the lung and the localization of laminin-5 $\gamma 2$ chain expression in intrabronchial precancerous lesions.

Materials and Methods

Materials

We included 57 cases of surgically resected SCC of the lung and 36 biopsy-resected intrabronchial lesions in the current study. These samples had been resected between 1995 and 1999 at the Department of Surgery, Tokyo Medical University Hospital. The patients ranged in age from 45 to 85 (mean 60.1) years. Each sample was diagnosed as stage I, II, or III disease. None of the patients had received chemotherapy or radiation therapy before surgery. Twenty-one cases (36.8%) were classified as stage I disease, 15 (26.4%) as stage II, and 21 (36.8%) as stage III. The biopsy samples were diagnosed pathologically as bronchial squamous metaplasia in 5 cases, dysplasia in 5 cases, carcinoma *in situ* in 4 cases, microinvasion to the bronchial wall in 1 case, and stage I intrabronchial squamous cell carcinoma in 21 cases.

Immunohistochemistry

The primary antibody used for immunohistochemical staining was mouse monoclonal anti-laminin-5 $\gamma 2$ [Ono Y. *Cancer*, 1999] (1-97; kindly provided by Dr. Setsuo Hirohashi, National Cancer Center Research Institute, Tokyo, Japan). Surgically resected specimens were fixed in 10% formalin and embedded in paraffin,

4- μ m-thick tissue sections were cut from each block, mounted on poly-L-lysine-coated slides, and dried overnight at 37°C. Sections were deparaffinized with xylene, treated with 0.3% hydrogen peroxide in methanol, and rehydrated in PBS. They were then autoclaved in 10 mM citrate buffer (pH 6.0) for 10 min at 120°C, and allowed to cool at room temperature for 30 min. The sections were preincubated in 2% normal porcine serum in PBS, and incubated overnight at 4°C with the primary antibody against laminin-5 $\gamma 2$. They were then washed three times with PBS and treated with an appropriate secondary biotinylated antibody (1:200 dilution, Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 30 min. Following several washes with PBS, the slides were reacted for 30 min with avidin-biotinylated horseradish peroxidase H complex, rinsed three times in PBS, and incubated for 1–3 min in 0.05% diaminobenzidine (DAB) including 0.01% hydrogen peroxide. The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. As positive controls for every assay, sections we had previously validated to be strongly positive were used. As a negative control, the same class of mouse immunoglobulin was used instead of the primary antibody.

Evaluation of laminin-5 $\gamma 2$ chain expression

Two observers (YE and HS), who had no previous knowledge of each case, independently reviewed the immunohistochemically stained sections, and all discrepancies were resolved by joint review of the slides in question. Biopsy samples were categorized according to the number of immunopositive tumor cells attached to the basement membrane as $-$, and $+$, indicating that less than 5%, and more than 5% of the tumor cells were positive, respectively. Surgical specimens were categorized according to the number of immunopositive tumor cells as $-$, $+$, and $++$, indicating that less than one twentieth, one twentieth to one fifth, and more than one fifth of the tumor cells were positive, respectively.

Statistical analysis

The relationship between laminin-5 $\gamma 2$ expression and clinicopathological factors was analyzed using the χ^2 method and Fisher's exact test. Survival rates were calculated by the Kaplan-Meier method for analysis of censored data. The significance of differences in survival was analyzed using the log-rank test by univariate analysis with the SPSS ver. 8.0 (SPSS Inc., Chicago, IL, USA) software package. Differences were considered significant at $P < 0.05$.

Results

Immunohistochemistry of the intrabronchial precancerous lesion

No tumor sample was completely negative. Overall, expression of laminin-5 $\gamma 2$ was observed in 2 of 14

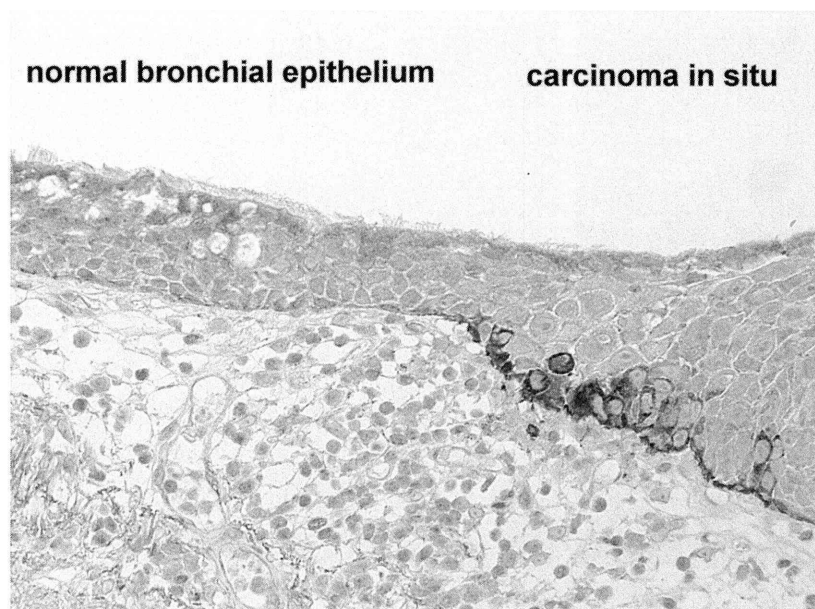


Fig. 1 Immunohistochemical staining of laminin-5 γ 2 in the border of carcinoma in situ and normal bronchial epithelium. Magnification : $\times 200$.

Table 1 Expression of laminin-5 γ 2 in intrabronchial lesions

	Bronchial squamous metaplasia	dysplasia	carcinoma in situ	microinvasion to bronchial wall	Intrabronchial squamous cell carcinoma
Laminin-5 γ 2 (-)	5 (100%)	5 (100%)	2 (50%)	0 (0%)	5 (23.4%)
Laminin-5 γ 2 (+)	0 (0%)	0 (0%)	2 (50%)	1 (100%)	16 (76.6%)
total (cases)	5	5	4	1	21

precancerous lesions (14.3%), 0 of 5 cases of bronchial squamous metaplasia (0%), 0 of 5 cases of dysplasia (0%), 2 of 4 cases of carcinoma in situ (50.0%), and in the only case of microinvasion to the bronchial wall (100%). Sixteen (76.6%) of the 21 cases of intrabronchial squamous cell carcinoma were positive for laminin-5 γ 2. These results are summarized in Fig. 1, 2 and Table 1.

Immunohistochemistry of squamous cell carcinoma of the lung

Expression of laminin-5 γ 2 was clearly demonstrated in SCC of the lung, but not in normal epithelial or stromal cells. All of the positive immunoreactions were cytoplasmic and were seen especially in cancer cells at the cancer-stromal interface, the staining intensity in each specimen being moderate to strong (Fig. 3). According to the number of tumor cells positive for laminin-5 γ 2, 27 (47.4%) of the 57 tumors were categorized as -, 12 (21.0%) as + and 18 (31.6%) as ++ (Table 2). Most of the cells that showed positivity for laminin-5 γ 2 in ++ tumors had proliferated into strands or small nests, whereas most of those in + or ++ tumors had grown into large tumor nests or sheet-like structures with or without small tumor nests.

The centers of all large tumor nests were negative for laminin-5 γ 2.

Association between the category of the number of immunopositive tumor cells and patient's prognosis

The increased number of immunopositive tumor cells was significantly associated with a reduced overall patient survival ($P=0.0151$; Fig. 4). In SCC of the lung, the patients with a low expression of laminin-5 γ 2 had a good prognosis when compared to those with high values.

Discussion

In this study, we examined the expression of the laminin-5 γ 2 chain in 14 intrabronchial precancerous lesions, 22 cases of intrabronchial squamous cell carcinoma and 57 cases of SCC of the lung. Overall, laminin-5 γ 2 expression was observed in 2 (16.7%) of the intrabronchial precancerous lesions, 17 (77.3%) of intrabronchial cancer lesion and 30 (52.6%) of the SCC of the lung. In most tumors, laminin-5 γ 2 was localized in the cytoplasm of tumor cells at the tumor-stromal interface. Focal but strong expression was found frequently in cancer cells that showed budding into the fibrous stroma. Intense staining for laminin-5 γ 2 was

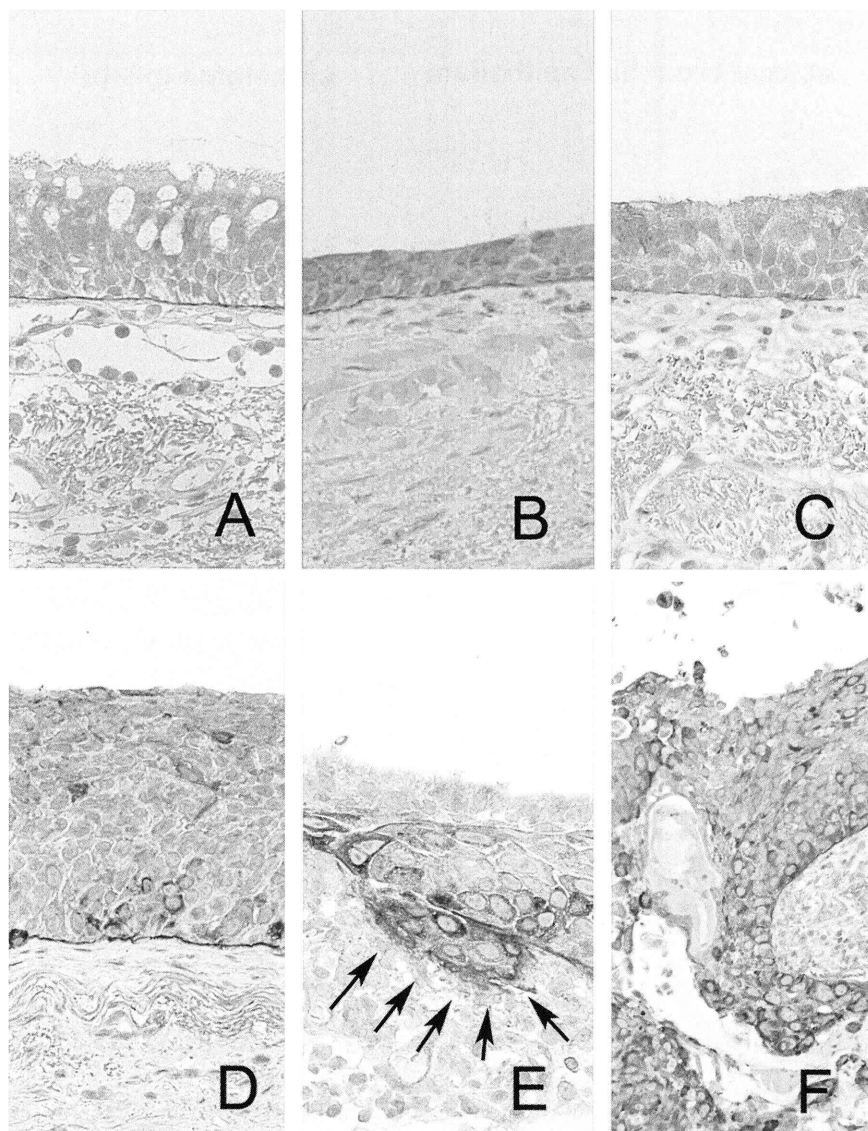


Fig. 2 Expression of laminin-5 γ 2 in the intrabronchial precancerous lesions.
 (A) Negative expression of laminin-5 γ 2 in normal bronchial epithelium.
 (B) Negative expression of laminin-5 γ 2 in squamous metaplasia.
 (C) Negative expression of laminin-5 γ 2 in dysplasia.
 (D) Positive expression of laminin-5 γ 2 in carcinoma in situ attached to the basement membrane.
 (E) Positive expression of laminin-5 γ 2 at the invasive front in microinvasion to the bronchial wall. Arrows indicate the invasive front.
 (F) Positive expression of laminin-5 γ 2 in intrabronchial squamous cell carcinoma.
 Magnification: $\times 400$ for A-F.

also found in cancer cells that had invaded the stroma in scattered, thin strands. These observations are in keeping with previous studies of colon cancer⁸⁾⁹⁾¹¹⁾¹⁵⁾, gastric cancer¹⁶⁾, pancreatic cancer¹⁷⁾, breast cancer¹¹⁾, uterine cervical cancer⁷⁾⁸⁾¹¹⁾, oral squamous cell carcinoma¹²⁾¹⁴⁾¹⁸⁾¹⁹⁾, and malignant melanoma⁸⁾¹¹⁾. The preferential expression of laminin-5 γ 2 at the invasive front of cancer indicates that laminin-5 γ 2 may be useful as a marker for stromal invasion by cancer cells. In this study, laminin-5 γ 2 expression was found in 16.7% of bronchial precancerous lesions. Although this observation may indicate a tendency for these lesions to

be invasive and/or prognostic potential, additional studies will be needed to determine the significance of these laminin-5 γ 2-positive cells in intrabronchial precancerous lesions.

An important step in the metastasis of cancer is the proteolysis of extracellular matrix proteins, which allows migration of neoplastic cells through the basement membrane into the interstitial stroma²⁰⁾. Human membrane-type-1 matrix metalloproteinase (MT-1-MMP) is a key enzyme in the process of extracellular matrix protein breakdown. It has been shown recently that rat laminin-5 can serve as a substrate for MT-1-

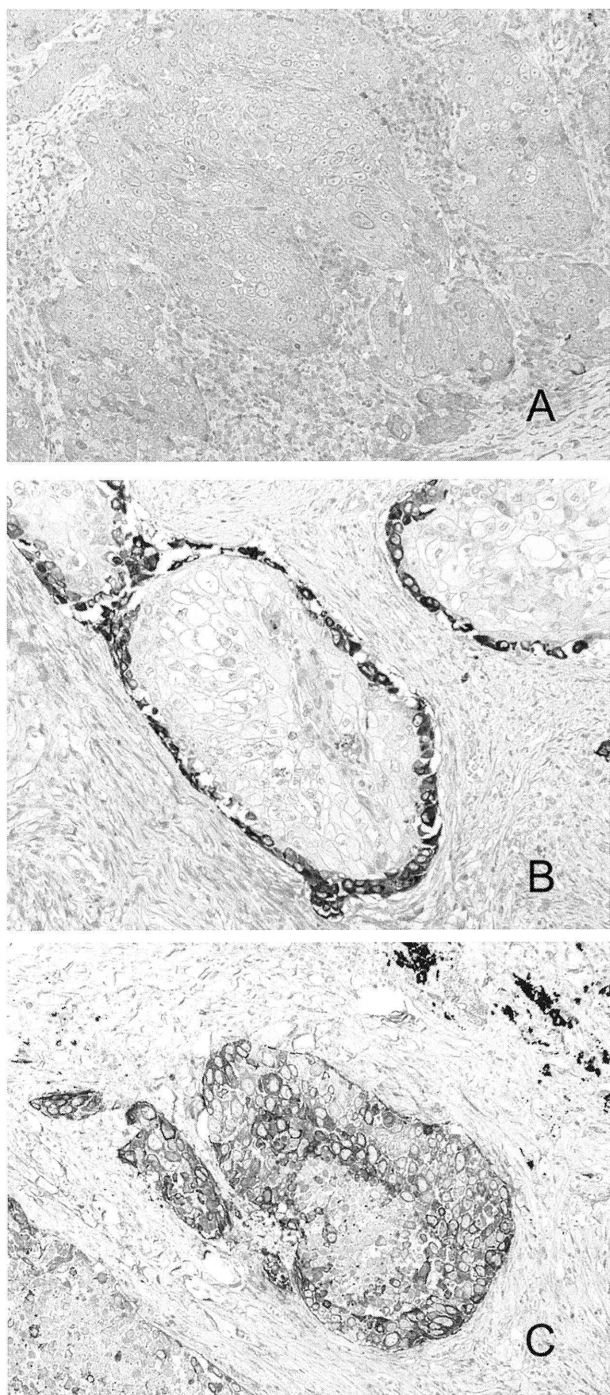


Fig. 3 Immunohistochemical staining of laminin-5 γ 2 in surgically resected SCC of the lung.
 (A) Expression (-) : few or no tumor cells were positive.
 (B) Expression (+) : the tumor nest was circumferentially positive.
 (C) Expression (++) : almost all tumor cells were positive. Magnification : $\times 200$ for A-C.

MMP and MMP-2²¹⁾²²⁾. It has also been reported that cell migration on laminin-5-coated Transwell filters is correlated with MT-1-MMP expression and can be abolished by MMP inhibitors. Thirupandiyur et al. examined the potential role of the MT-1-MMP-cleaved

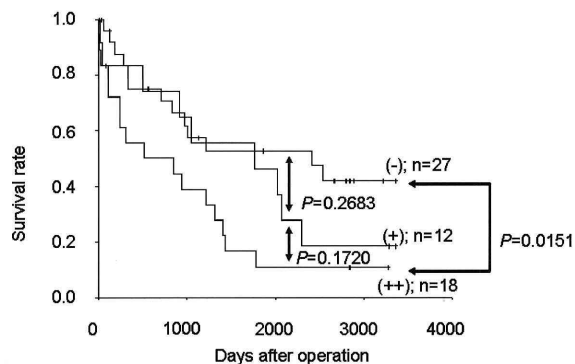


Fig. 4 Kaplan-Meier overall survival curve of laminin-5 γ 2 Chain expression are shown for 57 patients with squamous cell carcinoma of the lung (log-rank test for trend : $P=0.015$).

laminin-5 β 3 chain in the migration of DU-145 prostate carcinoma cells²³⁾. Their data suggested that the cells showed significantly increased migration on MT-1-MMP-cleaved laminin-5. A previous study has shown that tumor cells at the cancer-stromal interface and at the invasive front frequently co-express immunopositivity for the laminin-5 β 3 and γ 2 chains in both squamous cell carcinoma of the tongue and colorectal carcinoma. Strong expression of these two proteins was clearly demonstrated in cancer cells showing diffuse invasion²⁴⁾. Taken together, these findings indicate that not only laminin-5 γ 2 but also β 3 play an important role in the invasive properties of cancer cells.

We believe that the ability of cancerous cells to invade the bronchial wall is acquired in a sequential manner during carcinogenesis. It has been established that malignant transformation arises through an accumulation of genetic alterations. This stepwise transformation is known as multistep carcinogenesis. Lung carcinoma is the leading cause of cancer death worldwide²⁵⁾. The prognosis of patients with lung carcinoma is generally poor, and the overall 5-year survival rate is 17%²⁶⁾. This makes primary lung carcinoma one of the most malignant solid tumors, with a wide range of invasive and metastatic behavior. There is a high probability that alterations in the genotype of bronchial epithelial cells play a role in carcinogenesis. In this context, bronchial squamous metaplasia and dysplasia can be considered as precancerous lesions, and mutation of the P53 tumor suppressor gene, and deletion of chromosome 17p have been reported in such lesions²⁷⁻³⁰⁾, being initially evident in the basal layer³¹⁾. We have recently reported that downregulation of α -catenin and/or β -catenin, which may reflect dysfunction of the cadherin-mediated cell-cell adhesion system, is an important marker of atypia during carcinogenesis of the bronchial epithelium³²⁾. We hypothesized that not only molecules involved in cell-cell adhesion, such

Table 2 Association between laminin-5 $\gamma 2$ expression in the SCC of the lung and clinicopathological factors

Factors	Laminin-5 $\gamma 2$ (-) <i>n</i> =27	Laminin-5 $\gamma 2$ (+) <i>n</i> =12	laminin-5 $\gamma 2$ (++) <i>n</i> =18	Significance <i>P</i> value ²⁾
Gender				
Male	26	12	16	
Female	1	0	2	0.361
Histology ¹⁾				
Well diff. SCC	3	1	1	
Mod diff. SCC	17	7	11	
Por diff. SCC	7	4	6	0.954
Primary tumor size				
pT0,1	10	1	3	
pT2	13	9	10	
pT3	3	1	5	
pT4	1	1	0	0.210
Pathologic stage (pTNM)				
Stage IA	6	1	0	
Stage IB	5	3	6	
Stage II	8	3	4	
Stage III	8	5	8	0.371
Nodal involvement				
negative (pN0)	12	7	9	
Positive (pN1, 2)	15	5	9	0.722
Lymphatic invasion				
negative	13	7	10	
positive	14	5	8	0.804
Vascular invasion				
negative	16	5	8	
positive	11	7	10	0.480

1) Well diff. SCC, well differentiated squamous cell carcinoma; mod diff. SCC, moderate differentiated squamous cell carcinoma; por diff. SCC, poorly differentiated squamous cell carcinoma.

2) *P* values determined by the χ^2 test.

as cadherin, but also those involved in cell-substrate adhesion, such as laminin-5, play an important role in the invasive behavior of cancer cells. Therefore, in the present study, we investigated the expression of the laminin-5 $\gamma 2$ chain in intrabronchial precancerous lesions and SCC of the lung. We found that increased laminin-5 $\gamma 2$ immunoreactivity in tumor cells, suggesting a high propensity for invasion, was a significant marker of poor outcome in patients with SCC of the lung. Moreover, our findings indicated that laminin-5 $\gamma 2$ may play an important role in the progression of human intrabronchial lesions and squamous cell carcinoma.

In the intrabronchial precancerous lesions we detected laminin-5 $\gamma 2$ expression at the invasive front. Our data suggest that expression of laminin-5 $\gamma 2$ is a relatively early event in the genesis of bronchial squamous cell carcinoma, and that increasing histological atypia is

accompanied by a further increase in the expression of this molecule. Finally, an increased level of laminin-5 $\gamma 2$ in SCC of the lung might play an important role in local invasion beyond the basement membrane and progression to an advanced stage.

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肺扁平上皮癌における発癌過程での laminin-5 γ 2 の発現

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【要旨】 目的：肺門部扁平上皮癌の発癌過程における初期浸潤部の変化を明らかにする目的で、肺門部発生の前癌病変と Carcinoma in situ 及び扁平上皮癌のそれぞれの癌浸潤部について laminin-5 γ 2 の発現形式を比較検討した。さらに肺扁平上皮癌における laminin-5 γ 2 の発現と予後との相関性についても検討を行った。

Laminin は基底膜に存在する細胞外マトリックス成分で、細胞を基底膜に接着させ、組織の構築と維持、および細胞機能の調節に重要な役割を果たし、構造は α 鎖、 β 鎖、 γ 鎖の 3 種類のサブユニットから構成されている蛋白分子である。

Laminin-5 γ 2 の癌浸潤先進部における発現は胃癌、膵臓癌、舌癌、食道癌、肺腺癌病変などにおいて認められることが報告されているが、肺門部扁平上皮癌の発癌過程における発現形式や肺扁平上皮癌における laminin-5 γ 2 の発現と予後の相関性は報告されてない。

材料と方法：1995 年から 1999 年までの期間で、気管支肺生検によって得られた 14 例の肺門部前癌病変と、22 例の肺門部扁平上皮癌及び、手術切除 57 例の肺扁平上皮癌検体を用い、マウスモノクローナル抗体を用いた laminin-5 γ 2 の免疫染色法を行った。染色パターンを 3 段階に分類し、それぞれに臨床病理学的な検討を行った。

結果：Laminin-5 γ 2 は主に癌細胞の細胞質に発現を認め、肺門部前癌病変では発現は認められず、特に癌の浸潤先端部では基底膜に沿った発現を認めていた。また肺扁平上皮癌における laminin-5 γ 2 の発現は有意に予後と相関した ($p=0.0151$)。

また laminin-5 γ 2 は肺門部扁平上皮癌の発癌過程においても重要な役割をもつことが示唆された。