

# 67-Kilodalton Laminin Receptor Expression Correlates with Worse Prognostic Indicators in Non-Small Cell Lung Carcinomas<sup>1</sup>

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## ABSTRACT

Tumor samples obtained from 72 patients resected for non-small cell lung cancer were stained immunohistochemically using an immunoperoxidase method and the MluC5 monoclonal antibody specific for the 67-kDa laminin receptor. Sixty-one of 72 patients (84.7%) displayed a MluC5-positive reaction, which was usually localized in both the inner surface of the plasmatic membranes and the cytoplasm of neoplastic cells. When we compared the laminin receptor expression with clinicopathological and biological parameters such as histotype, grading, T status, N status, ploidy, proliferative activity, vessel invasion, and p53 protein accumulation, the following results were observed: (a) the mean expression of the receptor was higher in the group of patients with metastatic nodal involvement than in those with uninvolved lymph nodes ( $P = 0.02$ ); (b) a high Ki-67 score ( $>13\%$  of positive cells) was observed in tumors with a higher mean value of laminin receptor ( $P = 0.004$ ); (c) the tumors harboring neoplastic emboli in their vessels showed a higher laminin receptor immunoreactivity ( $P = 0.02$ ); and (d) a borderline association was found between the high mean value of laminin receptor immunopositivity and p53 accumulation in neoplastic cell nuclei ( $P = 0.05$ ). Our observations indicate that detection of high tissue levels of 67-kDa laminin receptor is associated with an invasive phenotype in non-small cell lung cancer and may provide further information in the biological characterization of this type of cancer.

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## INTRODUCTION

One critical event of the metastatic cascade is the crossing of the basement membranes by cancer cells when they: (a) leave the primary tumor site; (b) intravasate blood or the lymphatic circulation; and (c) extravasate at metastatic sites. It has been proposed that among the various cell surface proteins able to interact with laminin, an important component of basal membranes, the 67-kDa high affinity laminin receptor plays a crucial role during tumor invasion and metastasis.

Furthermore, the expression of the 67-kDa protein has been shown to have an important prognostic role in some human cancers, as reported recently by Martignone *et al.* (1) in breast carcinoma. The 67-kDa laminin receptors seem to be implicated in human cancer progression. As a matter of fact, several *in vitro* and *in vivo* data suggest a putative involvement of this receptor in the migration, invasion, and metastatic capability of human cells (2, 3), as demonstrated by the analysis of the laminin receptor expression in different types of human cancers (4, 5). However, up until now, the relation between this receptor and tumor behavior has not been clarified completely.

Owing to the availability of a monoclonal antibody (MluC5) recognizing the 67-kDa laminin receptor in formalin-fixed and paraffin-embedded tumor samples (6), we analyzed the MluC5 immunoreactivity in a series of NSCLCs<sup>3</sup> to check, on the one hand, the expression of the laminin receptor in this type of cancer, and on the other hand, the association between MluC5 immunoreactivity and clinicopathological and biological parameters, such as size, nodal status, proliferative activity, and p53 expression, which represent both conventional (7) and putative new prognostic factors (8-10) in this very aggressive and frequent group of cancers.

## PATIENTS AND METHODS

**Patients.** Seventy-two patients resected for NSCLCs were analyzed. The surgical resection included a radical lymph node dissection for all patients. Intrapulmonary, hilar, and mediastinal lymph nodes, including subcarinal and lymph nodes superior to the carina, were excised, respectively. Patients (67 males and 5 women, mean age of 62.8) received no adjuvant systemic chemotherapy or thoracic radiation. The pathological features of the surgical specimens were classified and staged according to the WHO criteria (11) and the Tumor-Node-Metastasis staging system (12).

**Immunohistochemistry.** Freshly resected tissues from 72 consecutive NSCLCs were collected for immunohistochemical analysis. The samples were immediately frozen in liquid nitrogen and stored in a  $-80^{\circ}\text{C}$  freezer until sectioning. One

<sup>3</sup> The abbreviations used are: NSCLC, non-small cell lung cancer; PAb, purified monoclonal antibody; BVI, blood vessel invasion.

Table 1 MLCu5 immunoreactivity in NSCLC: relation to clinicopathological parameters

Variables	No. of cases (%)	MLCu5 immunoreactivity	
		Mean $\pm$ SD	<i>P</i> <sup>a</sup>
Sex			
Males	67 (93.1)	28.5 $\pm$ 22.5	0.1
Females	5 (6.9)	14.1 $\pm$ 31	
Histotype			
Squamous	45 (62.5)	28.4 $\pm$ 21.1	0.6
Adenoca	24 (33.3)	25.5 $\pm$ 27.9	
Anaplastic large cells	3 (4.2)	21.6 $\pm$ 18	
Grading			
G1	13 (18.0)	25.2 $\pm$ 25.6	0.7
G2	33 (45.9)	29.6 $\pm$ 21.9	
G3	23 (31.9)	24.3 $\pm$ 22.3	
G4	3 (4.2)	25.8 $\pm$ 23.2	
T status			
T <sub>1</sub>	13 (18.0)	34.4 $\pm$ 19.4	0.4
T <sub>2</sub>	50 (69.5)	25.2 $\pm$ 22.5	
T <sub>3</sub>	9 (12.5)	30.5 $\pm$ 31.5	
N status			
N <sub>0</sub>	53 (73.7)	23.8 $\pm$ 20.3	0.02
N <sub>1-2</sub> <sup>b</sup>	19 (26.3)	37.8 $\pm$ 28.1	

<sup>a</sup> Unpaired *t* test.<sup>b</sup> N<sub>1</sub> patients = 8; N<sub>2</sub> patients = 11.

portion of the same samples was fixed in 10% formalin and routinely processed for paraffin embedding. The monoclonal antibody MLCu5, which reacts with the 67-kDa laminin receptor, was used. A small cell lung cancer sample was used as positive control (13). The proliferative activity of tumor samples was evaluated using Ki-67 monoclonal antibody (Dakopatts, Copenhagen, Denmark) in frozen sections. The nuclear accumulation of p53 was detected by PAb 1801 (Oncogene Science, Manhasset, NY), which recognizes an epitope between amino acids 32 and 79. The avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) was used for each antibody, after overnight incubation with each primary antibody at the different dilutions (1:100 for MLCu5, 1:200 for PAb 1801, and 1:50 for Ki-67, respectively).

**Immunohistochemical Evaluation.** MLCu5 immunoreactivity was evaluated by a semiquantitative method, counting the percentage of positive cells in a minimum of 1000 neoplastic cells. The same method was used to evaluate PAb 1801 and Ki-67 immunoreactivity.

**Flow Cytometry.** Flow cytometry was performed on nuclear suspension prepared from 50- $\mu$ m sections of formalin-fixed, paraffin-embedded tissue of primary lung cancers. To determine the percentage of tumor cells in the analyzed tissue, adjacent 4- $\mu$ m-thick histological sections were cut before and after the 50- $\mu$ m sections used for flow cytometry. We considered the value of 25–30% as a good percentage of neoplastic cells. Briefly, the sections were dewaxed by xylene, rehydrated through a sequence of 90, 80, 70, and 50% ethanol, washed twice in H<sub>2</sub>O<sup>d/d</sup> and minced in 1 ml of 0.5% pepsin (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl (pH 1.5) at 37°C for 30 min. Samples were filtered through a 30-mm-pore poly-

Table 2 Relationship between LR expression and biological parameters

Variables	No. of cases (%)	LR expression	
		Mean $\pm$ SD	<i>P</i> <sup>a</sup>
Ploidy			
Aneuploid	38 (71.6)	23.4 $\pm$ 21.1	0.08
Diploid	15 (28.4)	35.5 $\pm$ 27.1	
Ki-67 score			
$\leq 13$	38 (52.8)	20.3 $\pm$ 20.8	0.004
$> 13$	34 (47.2)	35.6 $\pm$ 23.4	
p53 score			
Negative	19 (29.2)	19.7 $\pm$ 22.2	0.05
Positive	46 (70.8)	30.1 $\pm$ 23.4	

<sup>a</sup> Unpaired *t* test.

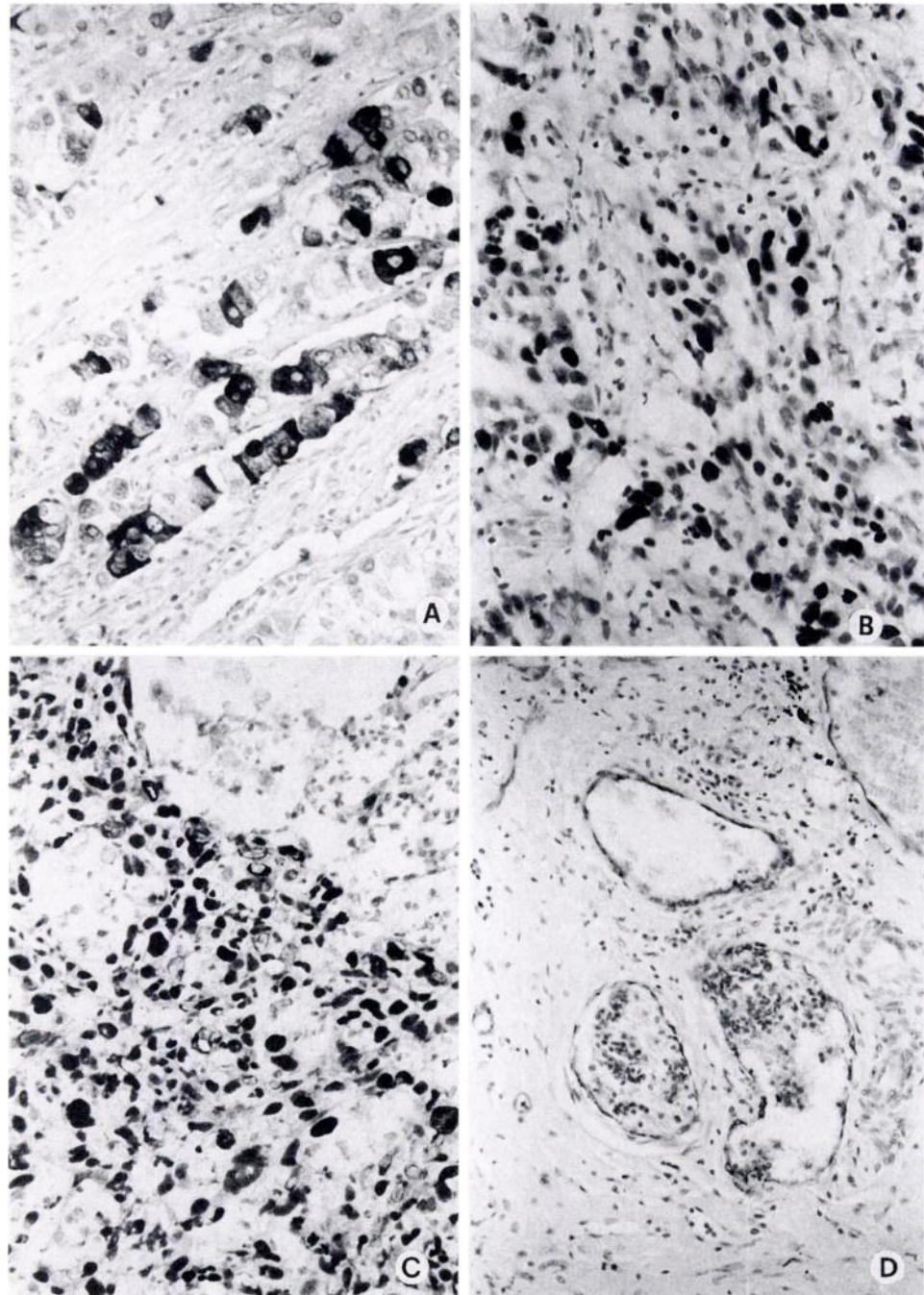
Table 3 Relationship between MLCu5 immunoreactivity and BVI in NSCLCs

Variables	No. of cases (%)	MLCu5 immunoreactivity	
		Mean $\pm$ SD	<i>P</i> <sup>a</sup>
BVI			
Negative	63 (87.5)	25.2 $\pm$ 21.6	0.02
Positive	9 (12.5)	43.8 $\pm$ 28.6	

<sup>a</sup> Unpaired *t* test.

ester filter and stained in a propidium iodide solution (50 mg propidium iodide/liter in PBS plus 0.1% v/v of NP40) for 30 min in the dark. Before analysis, the samples were syringed 2–3 times through a 25-gauge needle to avoid nuclear clumps. All samples were analyzed by a FACScan flow cytometer (Becton & Dickinson, San Jose, CA) coupled with a CONSORT 30 microcomputer (Becton & Dickson and Hewlett-Packard). For each sample, at least 35,000 events were acquired using the Cell Fit software (Becton & Dickson). Cell cycle analysis was performed by the Dean method, with no background subtraction. The two major drawbacks of paraffin-embedded material as a source of nuclei for DNA flow cytometry are the relatively poor resolution and the higher amount of cellular debris, which may affect the real percentage of S-phase fraction. Despite these potential limitations, in our study the histogram resolution was good, with 5% as the coefficient of variation median value of the diploid G<sub>0</sub>-G<sub>1</sub> peak. Moreover, to reduce the risk of missing some near-diploid tumors, histograms were considered interpretable only if the coefficient of variation of the diploid G<sub>0</sub>-G<sub>1</sub> peak was equal to or less than 7%. For each series of measurement, normal DNA fluorescence was adjusted to channel 200 by using nonneoplastic, paraffin-embedded lung tissue. In each measurement session (12–15 samples), we found that all tumor samples contained a stemline with normal DNA content. Aneuploidy was characterized by the presence of one or more additional stemlines, all with higher DNA content.

**Statistical Analysis.** Statistical analysis was performed by StatView II software. Unpaired *t* test and/or nonparametric



*Fig. 1* MLuC5 immunoreactivity (A) in the invasive component of a squamous NSCLC (ABC method;  $\times 250$ ); Ki-67 immunostaining (B) and p53 expression (C) in the nuclei of neoplastic cells (ABC method;  $\times 250$ ); and tumor emboli harboring within tumor vessels (D) highlighted by anti-FVIII monoclonal antibody (ABC method;  $\times 400$ ).

test (Mann Whitney *U* test and Kruskal-Wallis test) were used to evaluate differences between groups.

## RESULTS

**Clinicopathological and Biological Parameters.** The mean age of the patients was  $62.8 \pm 6.3$  (range, 46–79). As shown in Table 1, the most common histological type was squamous cell carcinoma (62.5%); 50 of 72 (69.5%) tumors were classified as T<sub>2</sub> (more than 3 cm on the greatest dimension), and 19 of 72 (26.3%) tumors showed metastatic nodal

involvement of hilar and/or mediastinal lymph nodes. 71.6% of the tumors had an aneuploid DNA content (Table 2). With regard to proliferative activity and p53 expression, 52.8% of the tumors showed a low Ki-67 immunoreactivity ( $\leq 13\%$  of positive cells), and 70.8% expressed p53 protein product (Table 2). Moreover, 9 of 72 (12.5%) tumors harbored tumor emboli in their vessels, whereas 63 did not (Table 3).

**MLuC5 Immunoreactivity According to Clinicopathological Parameters.** MLuC5 positivity was mostly localized in the membrane of the neoplastic cells, although slight immu-

noreactivity was also observed in the cytoplasm of the neoplastic cells (Fig. 1A). Sixty-one of the tumors (84.7%) were positive for MLuC5 monoclonal antibody (mean,  $32.8 \pm 22.2$ ; range, 1–80). The relationship between laminin receptor expression and clinicopathological parameters is reported in Table 1. Mean MLuC5 positivity was higher in tumors with metastatic nodal involvement than in those without metastases ( $P = 0.02$ ) at surgery. No correlations were found between MLuC5 immunoreactivity and other clinicopathological parameters such as histology, grading, and T status.

#### **MLuC5 Immunoreactivity and Proliferative Activity.**

The kinetic pattern of the tumors was evaluated by detecting the Ki-67 immunoreactivity of neoplastic cells. Positivity was confined to nuclei of neoplastic cells (mean,  $16.6 \pm 14$ ; range, 0.1–60%; Fig. 1B). We assumed the median value (13% of positive cells) as a cutoff value to distinguish tumors with low proliferative activity from tumors with high proliferative activity. Thirty-eight of the 72 neoplasms showed a Ki-67 immunoreactivity equal to or lower than the cutoff value, whereas 34 tumors presented a higher percentage of Ki-67-positive cells. As shown in Table 2, a good statistical association was observed between laminin receptor expression and proliferative activity, because the tumors with a high percentage of Ki-67 immunoreactive cells displayed a higher mean laminin receptor expression ( $P = 0.004$ ; Table 2).

#### **MLuC5 Immunoreactivity and p53 Protein Expression.**

p53 expression was detected in 46 (70.8%) of the 72 tumors analyzed (mean,  $44.7 \pm 27$ ; range, 1–90%; Fig. 1C). A higher laminin receptor expression was observed in p53-positive tumors compared with p53-negative ones, although a strong statistical association was not underlined.

#### **MLuC5 Immunoreactivity and Blood Vessel Invasion.**

Vessels in tumor samples were highlighted using a monoclonal antibody specific for the FVIII endothelial antigen (Fig. 1D). The presence of tumor emboli in endothelial-lined channels and their location in the specimen were assessed as follows: (a) intratumoral lymphatic vessel invasion or BVI, reflecting the presence of tumor emboli within the primary tumor; and (b) peritumoral lymphatic vessel invasion or BVI, which encompassed BVI peripheral to or at the advancing edge of the primary tumor. Lymphatic vessel invasion was distinguished from BVI, based on morphological characteristics reported previously (14). Tumors with BVI showed a significantly ( $P = 0.02$ ) higher laminin receptor expression (mean,  $43.8 \pm 28.6$ ) than those without (mean,  $25.2 \pm 21.6$ ; Table 3).

## **DISCUSSION**

The interaction of tumor cells with the basement membrane of different tissue types is one of the main determinants in local and distant dissemination (15). Cellular adhesion molecules, which mediate adhesion between cells and between cells and matrix, were identified. Laminin, a major component of the extracellular matrix, exhibits several biological properties including attachment, spreading, migration, proliferation, and differentiation of normal and neoplastic cells (16), as well as interactions between laminin and malignant cells, representing a crucial step during metastatic cascade. A growing number of identified cell surface proteins designed as laminin-binding pro-

teins and/or laminin receptors were characterized, and the 67-kDa is a cell surface protein binding laminin with high affinity. MluC5 monoclonal antibody was seen to recognize the 67-kDa laminin-binding protein (5) and was used to evaluate immunohistochemically different types of human solid cancers (1, 17–20).

In this study, we examined the laminin receptor expression together with some other clinicopathological and biological parameters that show a prognostic impact in NSCLC. Our results confirm the data obtained in other solid cancers (breast and colon; Refs. 1, 17, and 20) concerning the role of the laminin receptor in tumor development and metastatic progression. The association between laminin receptor expression and hilar and/or mediastinal nodal metastasis agree with the data by Martignone *et al.* (1) and by Liotta *et al.* (3), confirming that the laminin receptor may be considered a metastasis-associated protein. The influence of this protein on the metastatic process can also be inferred from the BVI results. As a matter of fact, a statistical association was observed between the laminin receptor expression and the presence of tumor emboli in the tumoral blood vessel. This data agrees with a study by Martignone *et al.* (1) in which an association was detected between MluC5 immunoreactivity and peritumoral lymphatic invasion, and it strongly supports the previous *in vitro* results of an involvement of this receptor in the invasive process (3).

The correlation between laminin receptor, proliferative activity, and p53 protein overexpression underlines the role of this protein in the metastatic progression of NSCLC. Because several studies have shown an association between bad prognosis and both high proliferative activity (21–23) and p53 protein alteration (9, 10) in different types of cancer including NSCLC, our results support the evidence that laminin receptors may identify a subset of cancers with a more aggressive behavior.

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