Laminin, Her2/neu and Ki-67 as prognostic factors in non-small cell lung cancer

Szelachowska J[1], Jeleń M[2]

[1] Department of Oncology, Medical University of Wrocław, Poland
[2] Department of Pathological Anatomy, Medical University of Wrocław, Poland

Abstract

Purpose: **The aim of this study was to determine the values of Ki-67 antigen, oncoprotein Her2/neu and laminin, as prognostic and predictive factors in NSCLC, and on the basis of these markers to create a prognostic model which would make it possible to identify patients with a high risk of disease recurrence.**

Material and methods: **The material for the study came from 64 patients with NSCLC, who underwent surgery in Dolnośląskie Centrum Gruźlicy i Chorób Płuc i 1996--2000, and subsequently were given radiation therapy in Dolnośląskie Centrum Onkologii.**

Results: **Among the markers researched, a high level of (intracellular) laminin in carcinoma cells was found to be an unfavourable prognostic factor. Also, another group of patients with an overexpression of oncoprotein Her2/neu were found to have a poorer prognosis, although the influence of proliferative index Ki-67 on patient survival could not be explained. The prognostic model LAMHER, which was defined on the basis of the intracellular laminin level and expression HER2/neu, enables the identification of a group of patients with a high risk of disease recurrence. In a multidimensional analysis of the "classification tree", it was found that patients with the highest risk of disease recurrence were those with LAMHER = 1 (overexpression Her2/neu and/or a high level of intracellular laminin), and patients with LAMHER = 0 but a Fractionation Dilution Factor higher than 1.57.**

Conclusions: **The conclusion of this study is that multiple molecular marker testing is necessary to detect an independent prognostic impact on survival and is therefore superior to single marker testing. Based on LAMHER testing, two groups of patients could be defined: a low-risk group (LAMHER = 0) and a high-risk group (LAMHER = 1) for failure of standarized treatment.**

Key words: laminin, Her2/neu, Ki-67, non-small cell lung cancer, postoperative radiotherapy.

ADDRESS FOR CORRESPONDENCE:
Szelachowska J
Department of Oncology
Medical University of Wrocław, Poland
Fax: +48 71 361 91 11
e-mail: szelachow@mp.pl

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Introduction

Lung cancer was the most common cause of cancer deaths in 1996 in the region of Lower Silesia, Poland. Lung cancer composed 10.8% of all malignancies among women, second only to breast cancer. Among men it was the most common type of cancer, at 28.1% among all cancer morbidity. Five-year survival among men reached 7%, and among women 12.1% [1]. Surgery remains the basic treatment modality and offers the best chance to cure patients with NSCLC. However, less than 1/3 of these patients are suitable for this kind of treatment; the results of their treatment are not the best, because even if it is possible to conduct radical surgery most patients are not free of the disease. According to the Jassem study, five-year survival following surgery was in stage IA – 66%, IB – 53%, IIB – 30%, and IIIA – 15% [2]. These not so optimistic results have generated a search for new prognostic factors which would allow us to define groups of patients who require more intensive anticancer therapy.

Material and methods

Material for the study came from 64 patients with primary non-small cell lung cancer, who were operated on in DCGiChP and postoperatively underwent radiation therapy in DCO from 1996 until 2000. Some of them had also postoperative chemotherapy. Histopathologic types of

***Table 1*. Histopathologic types of primary tumours and treatment modalities**

		Histopathology			
		squamous ca.	**adenoca.**	**large cell ca.**	**In total**
No of patients		24	23	17	64
Type of operation	wegde resection	0	1	0	1
	lobectomy	10	17	10	37
	bilobectomy	2	2	0	4
	pulmonectomy	12	3	7	22
No of chemotherapy cycles	0	14	9	5	28
	1-2	2	3	7	12
	3	5	9	5	19
	4-6	3	2	0	5
Fractionation of postoperative radiotherapy	df=2Gy	21	14	13	48
	df=2.66Gy	3	9	4	16

***Table 2*. Disease Free Survival (DFS) and Overall Survival (OS) in weeks from the date of the operation**

	Women	**Men**	**Medium (weeks)**		**Minimum (weeks)**		**Maximum (weeks)**		**Standard deviation**	
			DFS	**OS**	**DFS**	**OS**	**DFS**	**OS**	**DFS**	**OS**
Healthy	6	14	182	182	104	104	285	285	53	53
Distant progression	6	13	59	105	10	21	155	223	31.4	51
Loco-regional progression	2	7	95	129	44	62	175	209	52.4	54
Loco-regional and distant progression	1	5	68	98	27	56	143	153	42	38
Another cancer	0	2	96	141	54	92	138	190	59.3	69
Death without cancer progression	0	8	85	85	32	32	213	213	65	65
In total:	**15**	**49**	**107**	**130**	**10**	**21**	**285**	**285**	**69.5**	**63**

primary tumours and treatment modalities are indicated in *Tab. 1*. Immunohistochemical analyses were performed on paraffin blocks of resected lung tissue from 64 patients. Primary monoclonal antibodies to Ki-67, Her2/neu and laminin (DAKO Rabbit Anti Human Ki-67 Antigen N 1574 LSAB /control slides N 1574/, Rabbit Anti Human c-cerbB-2 Oncoprotein N 1629 LSAB /control slides N 1629/ Monoclonal Mouse Anri Human Laminin klon 4C7 Isotype IgG2a, kappa) were used. All immunohistochemical data were assessed twice for each patient, without prior knowledge of the patient's outcome. Proliferation index Ki-67 was determined by scoring the percentage of malignant cells with positive nuclear staining in five microscope fields, using the microscope Olympus BX – 50 and the computer program MultiScanBase 08.98. Her2/neu expression was estimated according to a semiquantitative four-stage grading system. Laminin expression was also evaluated using the semiquantitative system. Separately, on a three-grade scale the percentage of the cells with laminin expression (0%, <50%, >50% cells) was estimated, and separately on a four-grade scale was estimated the intensity of intracellular laminin staining (0-3 grade). The extracellular laminin expression was evaluated using a semiquantitative four-stage grading system (0-3 grade). Overall cancer-specific survival was defined as the period from the date of operation to the date of cancer-death. An observation was made at the last follow-up to determine if the patient was either alive or had died of a cause other than NSCLC. Disease Free Survival was defined similarly. Kaplan--Meier curves were calculated for each variable. The log-rank test and the Cox proportional hazards model were used to examine the relationship between cancer-specific survival, disease-free survival and various potential prognostic factors. The level of significance was set at $p<0.05$. The association of all markers with clinicopathological parameters was evaluated using the Chi-Square Test. Statistical analysis was performed using STATISTICA ver. 6. The analysis of classification trees, one of the most popular techniques in data mining, was performed using STATISTICA ver. 6.

Results

The results of 64 patients treated for NSCLC were analysed statistically. We examined the association between the following parameters and their influence on survival and disease-free survival-gender, age, type of operation, radicality of operation, pTNM, histologic subtype, grade of histologic malignancy, number of chemotherapy cycles, total dose of radiotherapy, dose intensity, fractionation dilution, proliferation index Ki-67, Her2/neu expression, intracellular laminin expression, and extracellular laminin expression.

Tab. 2 shows the mean time of DFS and OS. Index Ki-67 was estimated for 47 patients. The mean index Ki-67 was 28.6% (range 0.5 to 90%). The only weak correlation was between high expression Her2/neu and low index Ki-67, without statistical power. There was no difference in DFS and OS between groups of patients with index Ki-67 >25% and <25%.

Figure 1. **Disease free survival for patients with intracellular laminin in more than 50% and less than 50% of the cells (p=0.02)**

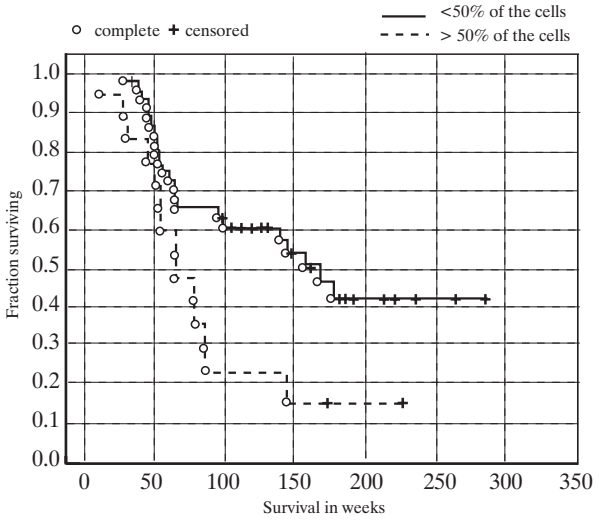


Figure 2. **Overall survival for patients with intracellular laminin in more than 50% and less than 50% of the cells (p=0.008)**

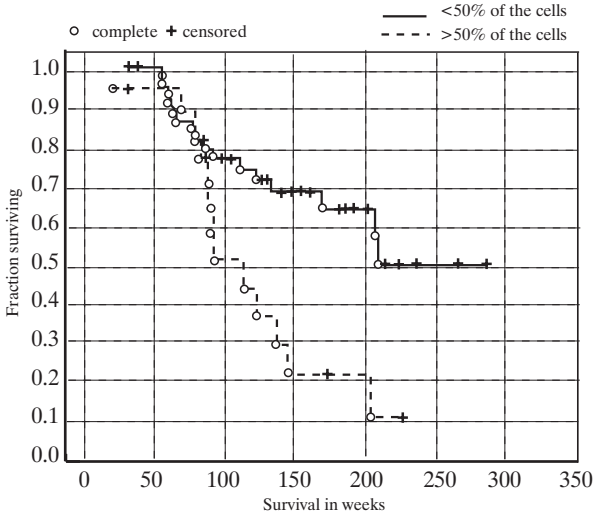


Figure 3. **Disease free survival for patients with the most intensive reaction against intracellular laminin and the rest of patients (p=0.02)**

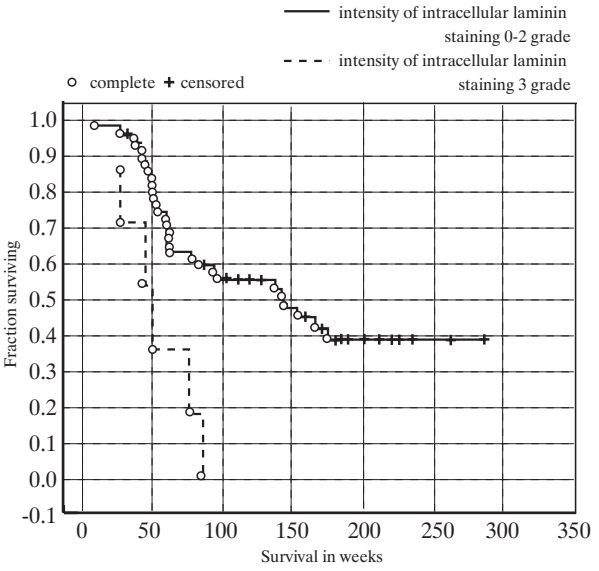
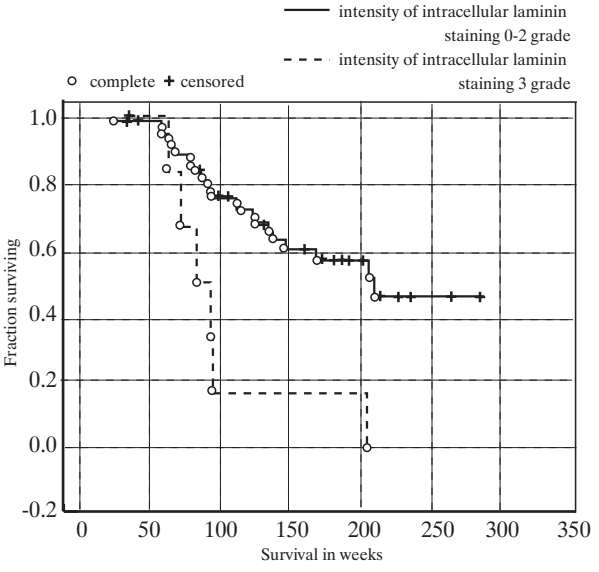


Figure 4. **Overall survival for patients with the most intensive reaction against intracellular laminin and the rest of patients (p=0.01)**



Her2/neu expression was evaluated for 64 patients, and was estimated as 0 for 21 (32.8%) patients, as 1 degree for 23 (35%) patients, as 2 degrees for 13 (20%) patients, and as 3 degrees for 7 (10%) patients. There wasn't any significant dependence between Her2/neu expression and other parameters.

DFS (*Fig. 1*) and OS (*Fig. 2*) were found to be statistically diminished among patients with laminin expression in more than 50% cancer cells. We found also to be statistically significant the difference in DFS (*Fig. 3*) and OS (*Fig. 4*) between patients with the most intensive reaction against intracellular laminin and the rest of the patients. There were no significant difference in DFS and OS on the other levels of extracellular laminin.

To identify groups of patients with poorer prognosis, we used tissue markers whose presence leads to a decrease of DFS – it was intracellular laminin and Her2/neu – whose influence on DFS was close to statistical significance. From among the 64 patients we constituted a group in which at least one of the chosen markers was on the highest level (Her2/neu overexpression or/and laminin in more than 50% cells or/and intracellular laminin staining intensity =3); and to simplify description in a subsequent analysis we introduced the term LAMHER. Comparison of curves of DFS (*Fig. 5*) and OS (*Fig. 6*) between groups of patients with at least one of the chosen markers on the highest level (LAMHER = 1) and the

***Figure 5*. Disease free survival for patients with LAMHER=1 and LAMHER=0 (p=0.0014)**

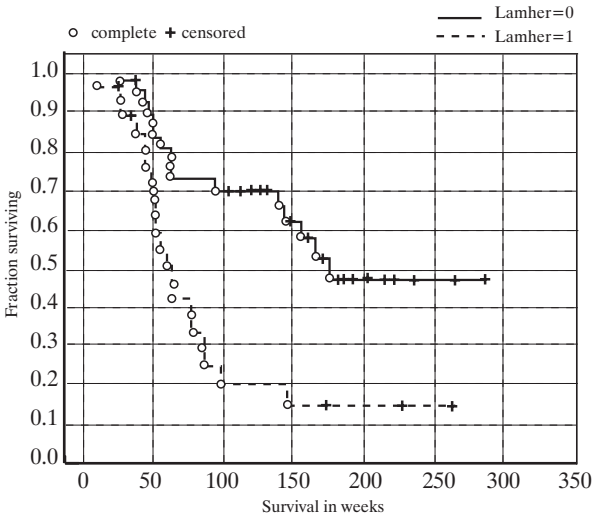


***Figure 6*. Overall survival for patients with LAMHER=1 and LAMHER=0 (p=0.00017)**

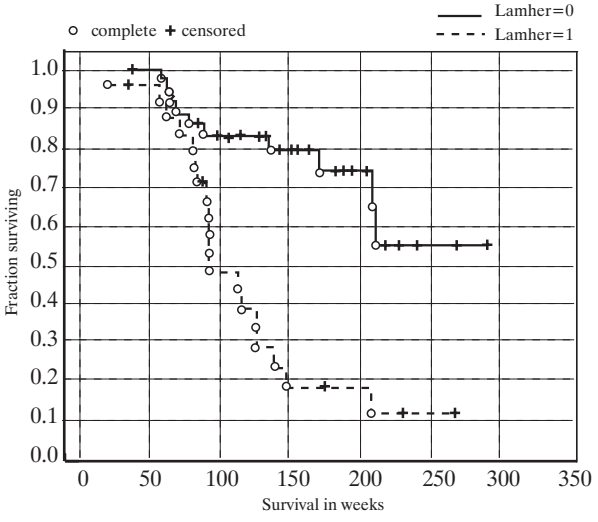
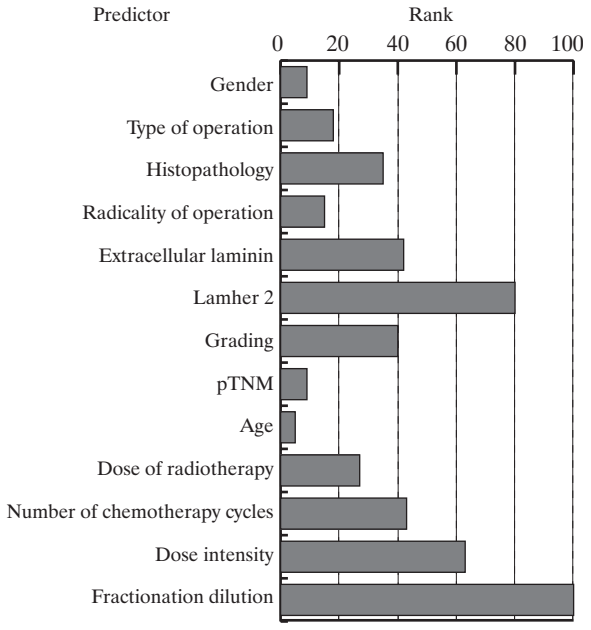


***Figure 7*. Rank of the parameters as predictive factors for reccurrence of the disease scale from 0 (low value) to 100 (high value)**



rest of studied patients (LAMHER = 0) showed a statistically significant difference.

Multivariate analyses was performed with the Cox proportional hazards regression model. From among the factors, only the level of the LAMHER factor was found to be the only independent risk factor (p=0.001, RR = 4.08).

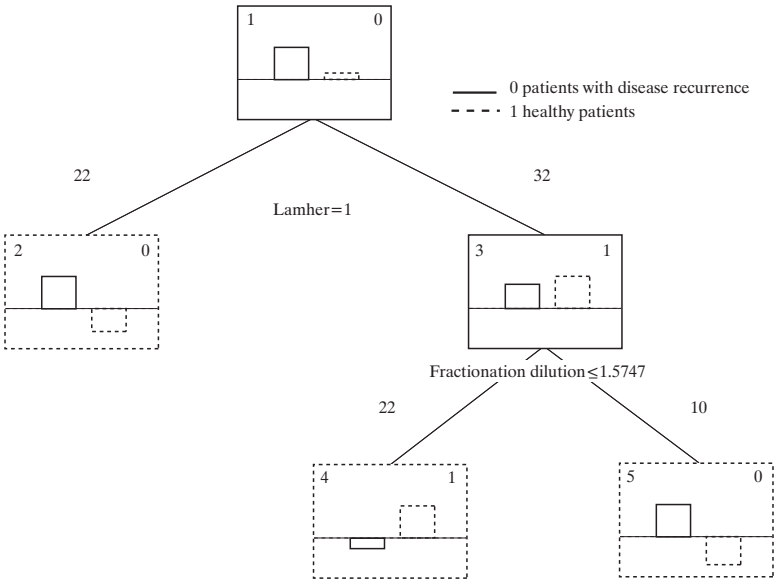
We used a multidimensional exploratory technique – analysis of classification trees – to find such predictive factors which would enable one, in the most exact and easiest way, to prognosticate recurrence of the disease. Analysis was based on observation of 54 patients; we excluded patients who died without cancer progression. *Fig. 7* shows the parameters taken into consideration in this analysis, and their rank as predictive factors. The most unfavourable prognostic factors according to a computerised algorithm were LAMHER and Fractionation Dilution (FD = overall time of radiotherapy/number of fractions radiotherapy). The scheme of the classification tree simply shows that the chance of curing patients with LAMHER equals 1 is close to 0, and for this group of patients the significance of the other factors is almost inessential. In the group with LAMHER equals 0 was the prevalence of patients without disease recurrence. Application of the Fractionation Dilution factor in this group enables one to distinguish between patients with better (FD≤1.57) and worse (FD>1.57) prognosis. Using this rule in our study, 10 of 54 patients were wrongly classified, six as healthy while they had recurrence of the disease, and four patients as ones with disease recurrence while in fact they were healthy. Global costs of faulty classification after the cross-matching test was 0.33.

Discussion

We designed this study to test the hypothesis that Her2/neu expression, the level of intracellular laminin and index Ki-67 by themselves or in combination are of prognostic importance in patients with resected NSCLC followed by postoperative radiotherapy. The markers in this study effect the malignant transformation and metastatic process through three oncogenic mechanisms: cell growth – Her2/neu, cell cycle regulation – Ki-67 and invasiveness – laminin.

We did not find a statistically significant difference between low and high proliferative indices. The relationship between Ki-67 expression and prognosis in NSCLC is still controversial.

Figure 8. **The tree classifying patients to high and low risk group for recurrence of the disease On the left side, there are histograms of the patients fulfilling the above-mentioned condition. Figures in the upper right corner mean which group of patients is in the prevalence**



However, most of the studies suggest decreased survival among patients with high Ki-67 expression [3-11]. In 2001 Ramnath showed limited value for the Ki-67 index, and concluded that Ki-67 index confidence intervals are large and weakly correlate with survival. He corroborated the importance of the Ki-67 index as a prognostic factor in patients with NSCLC is marginal [12].

Although the Kaplan-Meier survival analysis did not demonstrate the negative prognostic value of Her2/neu, there was a trend for poorer overall survival and disease free survival in patients with Her2/neu overexpression. However, a large number of patients would be necessary to prove a statistically significant effect of Her2/neu overexpression, more particularly as only 10% of our patients showed its overexpression. The major problem in comparing studies of Her2/neu expression in NSCLC is the enormous variations in frequencies of NSCLC tumours scored positive for Her2/neu, due to the application of different techniques and antibodies [13-27]. As a consequence different results were reported concerning the impact of Her2/neu overexpression on survival in NSCLC [16,18,21--23,28]. Most of the studies confirming a statistically important decrease of survival in patients with Her2/neu overexpression was performed on populations of patients with stage I NSCLC [3,17,18,24,29]. In the case of higher stage NSCLC, impact of the single marker on survival could be less distinct.

The factor with the strongest independent prognostic value was laminin. Kaplan-Meier overall and disease free survival analysis demonstrated the negative prognostic value of the intensive intracellular laminin expression and also the presence of laminin in more than 50% of cells. We used monoclonal antibody 4C7, which was acknowledged to be specific for the laminin a1 chain, but the latest studies have proved that it recognised rather the laminin a5 chain [30,31]. Most of the studies concerning the relation between laminin and cancer progression were based on laminin 5 (a3, b3, g2). In the case of this isoform, there was a dependence between laminin chains accumulation and malignant progression of the cancer [32-39]. There are very few publications concerning laminin in NSCLC [40-42].

To create a prognostic model we used molecular markers of two oncogenic mechanisms that have an influence on Disease Free Survival, namely intracellular laminin and Her2/neu, and we called these markers LAMHER. In the investigated group, only one patient had both Her2/neu overexpression and the highest level of intracellular laminin. We found a statistically significant difference between patients with LAMHER = 1 and the rest of the population in DFS ($p = 0.0014$) and OS ($p = 0.00017$). In the Cox analysis, the factor with the strongest independent prognostic value was LAMHER ($p<0.001$, RR = 4.08).

In the last years, a few studies were published in which the authors created biological risk models based on multiple molecular markers and showed that survival depends on the "dose" of cumulative unfavourable prognostic factors [3,17,24,29,43]. The conclusion of this study is that multiple molecular marker testing is necessary to detect an independent prognostic impact on survival and is therefore superior to single marker testing. Based on LAMHER testing, two groups of patients could be defined: a low-risk group (LAMHER = 0) and a high-risk group (LAMHER = 1) for failure of standarized treatment.

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