Prognostic Significance of Intracellular Laminin and Her2/*neu* Overexpression in Non-small Cell Lung Cancer

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Abstract. *This study was designed to test the hypothesis that Her2/neu expression, the level of intracellular laminin and Ki-67 index are of prognostic importance in patients with non-small cell lung cancer (NSCLC). The second aim of the study was to analyse the efficiency of post-operative radiotherapy in the group of patients treated at our hospital. Materials and Methods: Tumor tissue samples were obtained from 64 patients with primary NSCLC, who were operated on and post-operatively irradiated. Immunohistochemical analyses were performed on paraffin blocks of resected lung tissue. Survival of the patients was calculated on the basis of 5-year follow up. Results: From among the analysed factors only the enhanced expression of intracellular laminin, histological grade 3, adenocarcinoma type of cancer and statistically important radiotherapy parameters influenced patient survival. The influence of Her2/neu expression on survival almost reached statistical significance. In multivariate Cox analyses only the level of the intracellular laminin, overexpression of Her2/neu and dose time ratio (DTR) were found to be the independent risk factors for patients survival. Conclusion: Enhanced expression of intracellular laminin, Her2/neu overexpression and interruptions during post-operative radiotherapy are detrimental for survival in patients with NSCLC.*

Lung cancer is the most common worldwide cause of cancer deaths. Surgery remains the basic treatment modality and offers the best chance to cure patients with non-small cell lung cancer (NSCLC). However, 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. As matters stand, supplemental therapy is necessary in the majority of the patients. However, efficiency of post-operative radiotherapy is controversial (1-4). Despite the lack of definitive data, most experts continue to recommend the use of post-operative irradiation for patients with NSCLC in the N2 setting and after incomplete resection. Disappointing NSCLC treatment results have generated a search for new prognostic factors which would allow us to define groups of patients who require more intensive anticancer therapy. Laminin is a large glycoprotein of the basement membrane, composed of three sub-units: a heavy chain α and two lighter ones, β and γ. At present, at least 10 varieties of these chains and 11 laminin isoforms are known. Laminin plays an important role in the adhesion of cells to the basement membrane, in the extracellular matrix and in the ability of tumour cells to metastasise (5-11). As early as 1981, Albrechtsen *et al.* observed the presence of intracellular laminin in breast cancer cells in the areas of most active invasion and in the metastases to lymph nodes. They also observed that cancer cells with the ability to express laminin belong to selected clones characterised by high metastatic ability (12). Her2/*neu* is a transmembrane receptor with tyrosine kinase activity, containing intracellular, transmembrane and extracellular domains. Her2/*neu* promotes the transduction of proliferative and survival signals (13). Overexpression of Her2/*neu* was evident in a substantial group of human malignancies and indicated a poor clinical prognosis (13). The correlation between Her2/*neu* and outcome in NSCLC has been reported in several studies with controversial results (14). The most recent metaanalysis showed a significant, unfavorable prognostic effect of Her2/*neu* overexpression in NSCLC (15). The proliferation marker Ki-67, provides only limited information about the cell cycle status (16-18). Moreover, the usefulness of Ki-67 as a prognostic factor requires further evaluation since results of studies which have evaluated the relationship between cell proliferation and clinical course of the NSCLC are contradictory. This seems to some extent understandable since the function of the Ki-67 protein remains unknown and, in fact, the protein is not a key element in cell proliferation (19). Expression of Ki-67 may appear also when synthesis of DNA is blocked and in cells undergoing apoptosis (17).

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This study was designed to test the hypothesis that Her2/*neu* expression, the level of intracellular laminin and Ki-67 index are of prognostic importance in patients with resected NSCLC followed by post-operative radiotherapy. The second aim of the study was to analyse the efficiency of post-operative radiotherapy in the group of patients treated at our hospital.

Materials and Methods

Samples were taken from 64 patients with primary NSCLC, who were operated on in Lower Silesian Lung Disease Center (LSLDC) between 1996 and 2000. All patients were post-operatively irradiated in the Lower Silesian Oncology Center (LSOC), in case of microscopically incomplete resection or/and positive mediastinal lymph nodes. The interval from operation to radiotherapy ranged from 3 to 29 weeks as 35 patients received post-operative chemotherapy usually consisting of cisplatin with etoposide, or mitomycin with ifosfamide and cisplatin, before radiotherapy. Fractionation of radiotherapy was 2 Gy or 2.66 Gy per day, with five fractions per week; the total dose was 39.9 to 64 Gy. Because two different fractionation schedules were used, the normalized total dose (NTD) was recalculated using the linear – quadratic formula (where $\alpha/\beta = 35$ Gy for lung cancer) to enable comparison between total physical dose values (20). Furthermore, because of interruptions in radiotherapy caused by mechanical failure, loss of part of the dose was taken into consideration to defray repopulation, for treatments exceeding 28 days (21). We calculated the NTD with time correction (NTD-T) assuming a loss of 0.6 Gy per each day of the break. The dose intensity factor (DIF) (total dose/overall time of radiotherapy), the fractionation dilution factor (FDF) (overall time of radiotherapy/number of fractions) and the dose time ratio (DIF/FDF) which defines the degree of interdependency between the first two, were also calculated (22).

Immunohistochemical analyses. These were performed on resected lung tissue in paraffin blocks 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/ and Monoclonal Mouse Anti Human Laminin clone 4C7 Isotype IgG2a, kappa) were used. All immunohistochemical data were assessed twice for each patient, without prior knowledge of the patient's survival. The proliferation index for Ki-67 was determined by scoring the percentage of malignant cells, with positive nuclear staining, in five microscope fields, using an Olympus BX–50 microscope and the computer program MultiScanBase 08.98. Her2/*neu* and extracellular laminin expressions were estimated according to a semi-quantitative four-tiered grading system (0-3 grade). The percentage of the cells in which laminin was expressed was assessed on a three-grade scale: 0%, <50%, >50%, the intensity of intracellular laminin staining was assessed on four grade scale (0-3). Intracellular laminin was regarded as high when it was expressed in more than 50% of cells or/and the intensity of reaction was grade 3. Patient survival (disease free survival (DFS), overall survival (OS) and disease specific survival (DSS)) were calculated on the basis of follow up performed in both LSOC, LSLDC and using data from the Lower Silesian Cancer Register. Follow-up ceased in patients living for more than 5 years with no sign of progression of the disease. Statistical analysis was performed using STATYSTICA, version 6. 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 survival and various potential prognostic factors – gender, age, type of operation, radicality of operation, pTNM (stage of the disease defined pathologically), histological subtype, grade of histological malignancy, number of chemotherapy cycles, total dose of radiotherapy, dose time ratio, laminin, Her2/*neu* and Ki-67 levels. The association of all markers with clinical and pathological parameters was evaluated using the Chi-Square Test. The level of significance was set at $p<0.05$.

Results

Cumulative 5-year survival was 43% for DFS, 44% for DSS and 32.8% for OS. Table I shows the 5-year survival of patients in relation to specified clinical and pathological parameters. Of the analysed factors only enhanced levels of intracellular laminin significantly influenced patient survival. DFS, DSS (Figure 1) and OS were found to be statistically diminished among patients with enhanced intracellular laminin levels. Her2/*neu* expression was evaluated for 64 patients, and was estimated as 0 for 21 (33%) patients, as grade 1 for 23 (36%) patients, as grade 2 for 13 (20%) patients, and as grade 3 for 7 (11%) patients. The influence of Her2/*neu* expression on survival was close to statistical significance ($p=0.08$) Table I. The Ki-67 index was estimated for 47 patients with a mean of 28.6% (range 0.5 to 90%). The Ki-67 index did not affect patient survival. There wasn't any statistical significant correlation between Her2/*neu* expression, index Ki-67, intracellular laminin and other parameters. Of the other analysed factors only a high grade of histological malignancy (grade 3), cancer type (adenocarcinoma) and statistically important radiotherapy parameters influenced patient survival. Patients were divided into four groups (according to the level of NTD, and NTD-T): with dose levels of <44 Gy, 46-49 Gy, 50-54 Gy and >54 Gy. There was a significant decrease in survival in the groups of patients, who received the highest NTD (>54 Gy) and the lowest NTD-T (<45 Gy) dose of radiotherapy. Patients were divided into two groups according to DTR: 22 patients with DTR lower than 0.95, and 42 patients with DTR equal to or higher than 0.95. DFS, DSS and OS were found to be statistically diminished among patients with DTR <0.95 (Table I). There was no correlation between time from the operation to radiotherapy, overall time of radiotherapy, radicality of the operation or the pathological stage of the disease (pTNM) and survival of patients.

Multivariate analyses were performed with the Cox proportional hazards regression model. Only parameters which revealed statistical significance (or were close to) in univariate analysis were entered. Only the level of intracellular laminin and DTR <0.95 were found to be

Table I. *Five-year DFS (disease free survival), DSS (disease specific survival) and OS (overall survival) of patients in relation to specified clinical and pathological parameters.*

	No.	DFS	DSS	OS	*p*-value
Gender					
female	15	40%	47%	47%	NS
male	49	38%	43%	29%	
Age					
≤60	37	35%	44%	38%	NS
>60	27	45%	45%	26%	
Type of operation					
lobectomy	37	39%	41%	27%	NS
bilobectomy	4	75%	75%	25%	
pulmonectomy	22	39%	50%	45%	
pTNM					
I	5	60%	80%	60%	NS
II	20	52%	56%	45%	
III	39	27%	33%	23%	
Histopathology					
adenocarcinoma	23	25%	25%	22%	<0.05
other	41	46%	56%	39%	
Radicality of operation					
radical	6	66%	66%	66%	NS
uncertain radicality	32	40%	40%	31%	
incomplete operation	26	29%	43%	27%	
Number of chemotherapy courses					
0 cycle	28	36%	43%	29%	NS
1-6 cycles	36	41%	45%	36%	
NTD of radiotherapy					
<45 Gy	11	40%	40%	27%	<0.05
*45-49.9 Gy	2	0%	0%	0%	
50-54 Gy	43	49%	57%	42%	
>54 Gy	8	0%	0%	0%	
NTD-T					
<45 Gy	16	26%	27%	19%	<0.05
45-49.9 Gy	25	39%	47%	36%	
50-54 Gy	19	59%	65%	47%	
*>54 Gy	4	0%	0%	0%	
Ki-67					
<25%	25	40%	45%	32%	NS
>25%	22	37%	48%	36%	
Intracellular laminin					
low expression	44	48%	56%	45%	<0.05
high expression	20	17%	14%	5%	
Extracellular laminin					
low expression	54	43%	47%	35%	NS
high expression	10	20%	30%	20%	
Her2/*neu*					
Grade 3	7	14%	14%	14%	0.08
Grade 0-2	57	42%	49%	35%	
Grading					
Grade 3	16	13%	14%	16%	<0.05
other	48	40%	55%	47%	
DTR					
<0.95	29	14%	22%	17%	<0.05
≥0.95	35	58%	60%	46%	

*parameters not taken to statistical analysis.
NTD: normalized total dose; NTD-T: normalized total dose with time correction; DTR: dose time ratio; pTNM: post-operative pathologically assessed stage of disease; NS: non-significant.

Table II. *The results of multivariate Cox analyses for 5 years DFS, DSS and OS.*

	HR	*p*-value
DFS		
High level of intracellular laminin	2.3	0.02
Her2/*neu* overexpression	3.6	0.01
DTR <0.95	2.5	0.02
DSS		
High level of intracellular laminin	2.6	0.01
Her2/neu overexpression	2	0.04
DTR <0.95	2.5	0.04
OS		
High level of intracellular laminin	3	0.0009
DTR <0.95	2.1	0.047

DFS: disease free survival; DSS: disease specific survival; OS: overall survival; DTR: dose time ratio.

independent risk factors for DFS, DSS and OS (Table II). Additionally overexpression of Her2/*neu* was found to be an independent factor for DFS and DSS, but had no significant influence on OS.

Discussion

We did not find a statistically significant difference between low and high proliferative indices. The relationship between Ki-67 expression and prognosis in NSCLC remains controversial. However, most studies suggest decreased survival among patients with high Ki-67 expression (23-25). Although the Kaplan-Meier survival analysis demonstrated only a trend towards poorer survival in patients with Her2/*neu* overexpression, this parameter was found to be an independent risk factor for DFS and DSS. Conflicting results have been reported concerning the impact of Her2/*neu* overexpression on survival in NSCLC (15, 23, 26-30). The most recent metaanalysis showed a significant, unfavorable prognostic effect of HER-2 overexpression in NSCLC (15). The factor with the strongest independent prognostic value was laminin. Kaplan-Meier analysis of OS, DSS and DFS demonstrated a negative prognostic effect of intensive intracellular laminin expression and of the presence of laminin in more than 50% of cells. Monoclonal antibody 4C7, which was acknowledged to be specific for the laminin α1 chain was used in the present study, but the latest studies have proved that it rather recognized the laminin α5 chain (31, 32). Most of the studies concerning the relation between laminin and cancer progression were based on laminin 5 (α3, β3, γ2), and in the case of this isoform, there was a dependence between laminin chain accumulation and malignant progression (5-7, 11). There

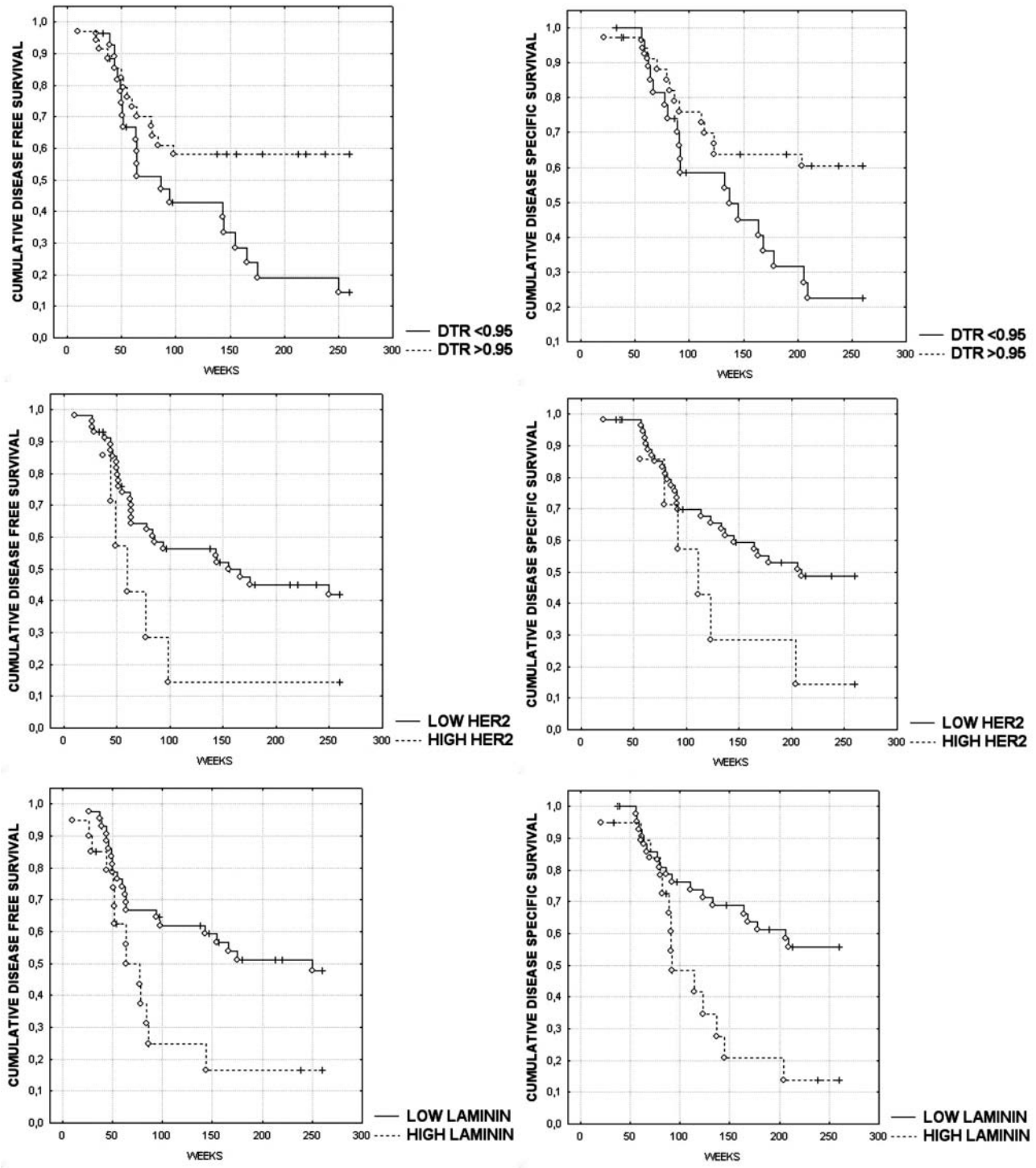

Figure 1. *Kaplan Meier curves for 5 years disease free survival and disease specific survival, according to the level of intracellular laminin, Her2/neu expression and DTR (dose time ratio).*

are very few publications concerning laminin in NSCLC and none is using 4C7 antibody (8-10). According to our knowledge this is the first study using 4C7 antibody in NSCLC.

A slight effect of the level of the NTD or NTD-T on patient survival was observed, but this was not confirmed in Cox multivariable analysis. However, we suggest that these

results may be misleading, especially since factor α/β taken into consideration in these calculations was not surely reliable and since the dose equivalent of repopulation was based on records from head and neck cancers. For these reasons, to compare the results of different fractionations of radiotherapy (different doses per fraction and interruptions during treatment), we used Maciejewski's concept, of simple factor – DTR (22). A decrease of DTR was associated with worsening of DFS, DSS and OS, survival decreases when there are breaks in the course of radiotherapy. Based on these results, it could be assumed that the time-table of radiotherapy is more important than the level of the total dose. However, the negative result of the connection between the total dose (NTD, NTD-T) and effect of radiotherapy (patients survival) should be treated with caution especially since there is a lack of credible calculations of the α/β factor and the dose-equivalent of repopulation for different histological subtypes of lung cancer.

The results from some studies have indicated the importance of the time factor in radical radiotherapy of lung cancer, and also confirmed that prolonged treatment time was adversely associated with survival (33-36).

Conclusion

Enhanced expression of intracellular laminin and Her2/*neu* overexpression are independent prognostic markers for reduced survival time. This paper shows also, that interruptions during post-operative radiotherapy are detrimental for survival in patients with NSCLC.

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