Glycohistochemical and microvascularization prognostic factors in lung cancer

Ph.D. Thesis

by

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ABBREVIATIONS

BC: binding capacity
CI: confidence interval
ECM: extracellular matrix
EX: expression
Gal-1: galectin-1
Gal-1-BC: galectin-1-binding capacity
Gal-1-EX: galectin-1 expression
Gal-3: galectin-3
Gal-3-BC: galectin-3-binding capacity
Gal-3-EX: galectin-3 expression
HA: hyaluronic acid
HA+Ca^{++}: hyaluronic acid in calcium-containing medium
HBL: heparin-binding lectin
MS: median survival time
NSCLC: non-small cell lung cancer
SCLC: small cell lung cancer
Sv: surface fraction
Vv: volume fraction

1. INTRODUCTION

The malignant tumour that currently occur most frequently and causes the death of the most patients in lung cancer. During the past 30 years, its incidence has increased more than 4-fold. In Hungary, 2530 new cases were recorded in 1970, wich correspond to an incidence of 14.5 per 100,000. The number of cases discovered in 2002 was 6361, *i.e.* an incidence of 62.4 per 100,000. In spite of the developments in the surgical techniques and radicality, the appearance of new cytostatic drugs and ever more up-to-date radiotherapy treatment possibilities, the management of lung caner is not solved and the efforts to date have been accompanied by only modest results: while the 5-year survival rate of lung cancer patients overall in the 1960s was 5%, with the development of (or despite) multimodal treatment the rate has risen during 40 years to only 14-15% (1). A better prognosis determination, and hence a more correct indication of multimodal treatment, may furnish a possibility for improvment of the results of the therapy of lung cancer. The structures and functions of numerous molecules are now known wich play a role in tumour progression (proliferation markers, oncogenes, tumour suppressor genes, growth factors, apoptosis markers and vascularization). Their study not only leads to a better understanding of the spreading of tumours, but may also permit a prognosis of the tumorous disease. Through a combination of the prognostic factors characterizing the various processes, it may be possible to set up substaging systems (2).
During the past 10 years, besides the earlier information-carrying molecules such as proteins, nucleic acids and a small number of steroids, increasingly more has been learnt aboit complex carbohydrates. Via their covalent binding with proteins or lipids, these account for a relatively high proportion of the cell surface; accordingly, they are in an ideal situation to participate in cell-cell and cell-matrix interactions. In response to differentation, tumorous degeneration or certain molecules, *e.g.* cytokines or steroid hormones, the carbohydate epitopes of the glycoconjugates may undergo quantitative and qualitative changes, and this may play a part in the regulation of the above-mentioned process of progression (3).
Investigation of the lectins contributes to a better understanding of the individual steps of tumour progression. The *in vitro* experimental results have already confirmed a number of

effects of the lectins on cell growth and tumour progression; these affect cell proliferation, the adhesion abilty of the cells, angiogenesis and the development of metastases (4-8).
Appropriate nutrient and oxygen supplies are required for the growth of tumours. The vessels needed for this may be autogenous vessels in the host organism itself, or they may be new vessels formed in response to angiogenetic factors produced by the tumour cells. The tumour cells cause the basement membrane of the proliferating capillaries to undergo damage and fragmentation and it thereby becomes possible for the migrating tumour cells to enter the blood circulation by migrating in the direction of lowest resistance (9). The prognostic value of angiogenesis was first described by *Macchiarini et al.* for non-small cell lung carcinoma (NSCLC) (9), and that finding has subsequently been confirmed by other authors (2, 11, 12).

2. AIMS

1. Can correlations be demonstrated between the pT, pN classification of lung cancer, the histological type and the hyaluronic acid-binding capacity, lectin-binding capacity and lectin expression of the tumours?
2. Do the hyaluronic acid-binding, lectin binding-capacity and lectin expression detected in lung tumour cells correlate with the survival, and does this have a prognostic role?
3. Does syntactic structure analysis reveal correlations between the pT, pN classification of lung cancer, the histological type and the tissue structure of lectin-binding and lectin-expressing tumours?
4. Does the structure of hyaluronic acid-binding, lectin-binding and lectin-expressing lung tumours, characterized by syntactic structure analysis, correlate with the survival in radically operated patients, and does this have a prognostic role?
5. Do the quantitative parameters of the vascularization of tumour correlate with the pT, pN classification of lung cancer and the histological type?
6. Does the vascularization of lung cancer influence the survival of radically operated patients?

3. QUALITATIVE AND QUANTITATIVE LECTIN-IMMUNOHISTOCHEMICAL PARAMETERS OF LUNG CANCER AND THEIR CORRELATION WITH THE PROGNOSIS

3.1. Patients and methods

Histological studies were performed on 481 patients who underwent radical surgery for lung cancer between 1 January 1990 and 31 December 1995. 246 of these patients were operated on in the thoracic surgery unit at the Thoraxklinik in Heidelberg (Germany), and 235 at the Department of Surgery, University of Szeged. The average age of the patients was 58.7 years. There were 390 men and 91 women. The histological types were distributed as follows: sqamous cell carcinoma: 216 (44.9%), adenocarcinoma: 178 (37%), large cell carcinoma: 67 (13.9%), small cell carcinoma: 20 (4.2%). The tumours were in the following stages: I/A: 60 (12.5%), I/B: 178 (37%), II/A: 6 (1.2%), II/B: 91 (19%), III/A: 137 (28.5%), III/B: 6 (1.2%) and IV: 3 (0.6%). No statistically significant difference ($p>0.05$) was seen when analyzing these data for the two different centres with exception of age.
Sections, 4-6 μm thick were prepared from the paraffin embedded tumour tissue and a immunohistochemical staining technique was peformed. The lectin-binding capacities of the tumour cells were studied with the aid of labeled (biotinylated) galectin-1 (Gal-1), galectin-3 (Gal-3) and CL-16. The investigation of the hyaluronic acid-binding capacity was carried out with biotinylated hyaluronic acid molecules prepared in a conservation medium that was

either Ca^{2+}-free (HA) or contained 8 mmol/l Ca^{2+} (HA+ Ca^{2+}). The final dilution of all probes measured 10 µg/ml for 60 minutes. The visualization of the binding capacities was undertaken in relationship to the routine immunohistochemical staining technique, and was performed with the avidin-biotin technique (Vector Laboratories, Burlingame, USA), using diaminobenzidine (DAB) chromogen.
For the demonstration of lectin expression, IgG antibodies against galectin-1-, galectin-3- and heparin-binding lectin (HBL), produced in rabbit, were used. Antibody binding was detected with the use of monoclonal IgG against rabbit immunoglobulin, with a streptavidin-biotin method as the labelling system (BioGenex, San Ramon, USA). Counterstaining was performed to label the nuclei of the tumour cells. Both positive and negative controls were performed in staining slides with known immunohistochemical staining, and by omission of the primary antibody or lektin.
During the routine light-microscopic examinations, the sections were all evaluated by the same pathologist, who classified them as negative or positive on the basis of the intensity of the staining.
Tumours that exhibited staining were subjected to syntactic structure analysis by means of computerized image analysis. Images were processed with the aid of DIAS software (Towersoft, Berlin, Germay), for wich the user's program was written at the pathology unit at the Thoraxklinik in Heidelberg. In the first step, the centres of the nuclei of the tumour cells and of the lymphocytes observed in the tumour tissue were labelled in an interactive manner. At least 300 tumour cells and 50 lymphocytes were labelled in each section.
A cluster was defined as a group of cells where the intercellular distance (d_0) did not exceed the sum of the main intercallular distance (m) and twice the standard deviation (sd): ($d_0 \leq m(e_0) + 2sd(e_0)$). The structural entropy was determined by the method of *Kayser* (13), with use of the differences in distance and staining between the cells:

$$\mathbf{E(MST) = \Sigma[(D_i(Ic)/Ic]^2+[D_i(r)/r]^2,}$$ where

$D_i(Ic)$ = difference in staining between the closest neighbouring cells,
Ic = mean staining of all cells
Di(r) = distance between the closest neighbouring cells, and
r = mean distance bwetween the cells

In the course of the syntactic structure analysis, the following parameters were determined:
1. The proportion of unstained tumour cells (%)
2. The proportion of moderately stained tumour cells (%)
3. The proportion of intensely stained tumour cells (%)
4. The mean distance between tumour cells (µm)
5. The mean distance between unstained tumour cells (µm)
6. The mean distance between moderately stained tumour cells (µm)
7. The mean distance between intensely stained tumour cells (µm)
8. The mean distance of lymphocytes (µm)
9. The mean distance of unstained tumour cells from lymphocytes (µm)
10. The mean distance of moderately stained tumour cells from lymphocytes (µm)
11. The mean distance of intensely stained tumour cells from lymphocytes (µm)
12. The mean number of tumour cells per clusters
13. The mean number of unstained tumour cells per clusters
14. The mean number of moderately stained tumour cells per clusters
15. The mean number of intensely stained tumour cells per clusters
16. The mean diameter of clusters of tumour cells (µm)
17. The mean diameter of clusters of unstained tumour cells (µm)

18. The mean diameter of clusters of moderately stained tumour cells (μm)
19. The mean diameter of clusters of intensely stained tumour cells (μm)
20. The structural entropy

The results were compared with the pT and pN status, the histological findings and the survival data. The data were subjected to statistical processing with the chi-square test and ANOVA. The *Kaplan-Meier* method and *Cox* regression analysis were used for the survival calculations; in the former method, significance was established with a log-rank method.

3.2. Results

3.2.1. Correlation of the qualitative lectin histochemical results with the pT, pN classification and the histology

In the event of more advanced lymph node metastases, the proportion of Gal-1-binding, Gal-3-binding and Gal-1expressing tumours displaysa discrete, but statistically not significant increase. Gal-3-EX is more frequent for pN0 tumours than for pN1-2 tumours. A the pT status advances, the proportion of tumours with Gal-1-BC decreases, while the proportion of tumours with Gal-1-EX gradually increases. In contrast, as the pT status increases, the proportion of Gal-1-binding and Gal-3-expressing tumours decreases, to a significant extent (p=0.001). The CL-16-BC does not demonstrate a correlation with either the lymph node status or the pT classification. The greatest proportion of HBL postivitiy is to be seen for T2 tumours; the number of positive cases for T1 and T3 tumours are around 10% smaller (p=0.006). The lymph node metastases did not display a statistically significant correlation with the HBL-EX.

The proportion of HA-binding tumours is higher in the event of more advanced lymph node metastases, but the difference is not significant. In teh more advanced pT stages, the proportion of Ha-binding tumours decreases, though not significantly, in both the Ca^{++}-free medium and the HA+Ca^{++} medium.

In every examination series, the proportion of cases exhibiting staining was substantially lower for the SCLC patients than for the NSCLC patients. For 5 examination series (Gal-1-EX, Gal-3-BC, Gal-3-EX, HBL-EX, and CL-16-BC), the difference was statistically significant. However, such a difference may also be detected between other groups. Thus, with exception of Gal-1-EX, the proportion of positive cases is essentially higher among the adenocarcinoma patients than in the squamous cell carcinoma or large cell carcinoma group. The largest difference was seen for the Gal-3-BC and Gal-3-EX (~15% and ~20%, respectively), and for the Gal-1-EX (p=0.022) and CL-16 (p=0.043) too statistically significant differences were observed in the distribution of the staining between the individual subgroups within the NSCLC group.

3.2.2. Correlation of the qualitative lectin immunohistochemocal results with the survival.

The median survival time (MS) in cases with tumours not expressing Gal-1 is 48.8 months, while in the event of positive staining it is 35 months (p=0.027). For Gal-3-binding tumours, MS is 35.3 months in the postive cases, and 45.9 months in the negative cases (p=0.039). In the event of Gal-1-BC and Gal-3-EX, MS is similarly better for the cases with negative-staining tumours, but the difference between the negative and positive cases did not proven significant. For Gal-1-binding tumours, MS is 40 months, while in the Gal-1-negative cases it is 46 months (p=0.368), for Gal-3-expressing tumours, MS is 42 months, while in the non-staining cases it is 44 months (p=0.255). For CL-16-binding tumours, MS is 40 months, while for tumours without CL-16-BC it is 46 months (p=0.391). The survival is better for HBL-

expressing tumours (MS=44 months) than for the negative cases (MS: 29.2 months), but the difference is not significant statistically (p=0.508). The HA-BC itself impairs the chances of survival (MS: 33.6 months vs. 44 months), while in the HA+Ca^{++} medium the survival for cases with HA-binding tumours exceeds that for the negative cases (MS: 51 months vs. 40.6 months). The difference in the survival is not significant.
In a multivariate analysis involving the classical prognostic factors (pT, pN and histology) and the lectin immonohistochemical results, the histology (p=0.003), pT (p=0.003), pN (p<0.001), the Gal-3-BC (p=0.01, RR: 0.736) and the Gal-3-EX (p=0.03, RR: 0.761) proved to be independent prognostic factors. The relative risk too was taken into considaretaion, when this connection indicated a similar prognostic strenght for the pT and pN classification Within the NSCLC group, pT (p=0.007), pN (p<0.001) and the Gal-3-BC (p=0.011, RR: 0.734) proved to be independent prognostic factors.

3.2.3. Correlation of the quantitative lectin immunohistochemical results with the pT, pN classification and the histological type

For Gal-1-binding tumours, in the event of a more advanced pT the proportion of intensely stained tumour cells progressively decreases to a significant extent. The distance between the intensely stained cells increases significantly in the more advanced pT stages. The structural characteristics of the Gal-1-binding do not display a correlation with the appearance of lymph node metastases. Both distance between the moderately stained tumour cells and that between intensely stained tumour cells differ significantly in the different histological types. The smallest distances were measured in the SCLC cases, and the largest intercellular distance in large cell carcinoma. The highest number of cells per cluster was found in the SCLC cases. The diameter of the cell clusters was least in the cases in the cases with SCLC, and for the moderately stained tumour cells this difference proved significant.
For Gal-3-binding tumours, none of the parameters exhibited a clear-cut decrease or increase as the pT stage progressed. A significant difference was found in the number of cells in the clusters formed by the moderately stained tumour cells, where the mean number of cells was highest in the pN1 cases. The proportion of intensely stained tumour cells was highest in cases of sqamous cell carcinoma, and significantly lower in cases of adenocarcinoma and large cell carcinoma. The value of the entropy was highest in the adenocarcinoma cases, somewhat lower in those with large cell carcinoma, and lowest in those with squamous cell carcinoma. The mean distance between the unstained tumour cells and the lymphocytes was about 3 times larger in the large cell carcinoma cases than in the adenocarcinoma cases (p=0.029).
For Gal-1-expressing tumours, the distance between the tumour cells increased significantly (p=0.026) as the pT stage progressed. An increase was also observed in each of differently stained subgroups, but not to significant extent. In stages pT2 and pT3, the number of cells per cluster decreased significantly both overall (p=0.016) and for the moderately stained tumour cells (p=0.028). Only the increasing diameter of the tumours cell clusters demonstrated a significant correlation (p=0.034) with the lymph node status.
The proportion of negative tumours cells was higher among the cases with more undifferentiated large cell carcinoma than among those with squamous cell carcinoma or adenocarcinoma (p=0.019). The distance between the tumour cells was least in the squamous cell carcinoma cases, and significantly higher in those with adenocarcinoma or large cell lung carcinoma (p=0.001). A similar tendency was observed for the distance between the moderately stained tumour cells (p=0.001). The diameters of the tumour cell clusters (p=0.011) and the unstained tumour cell groups (p=0.002) were substantially hogher in the large cell carcinoma cases than in those with squamous cell carcinoma or adenocarcinoma.

The value of entropy was lower in the squamous cell carcinoma cases than in those with adenocarcinoma or large cell carcinoma, where the values were similar (p=0.029).
For Gal-3-expressing tumours, it may likewise be observed that the distance between the tumour cells increases with increase in pT. This is also the situtation for the moderately and intensely stained tumour cells, but not to a significant extent. The entropy is significantly less in the lymph node-negative cases than in those with lymph node metastases (p=0.021). The mean number of cells in the intensely stained tumour cell groups was highest in the cases with N1 metastases, and lowest in the cases without lymph node metastases (p=0.034). in the NSCLC cases, the distances between the tumour cells and the moderately or intensely stained tumour cells were lowest for squamous cell carcinoma; greater distances were found in adenocarcinoma and large cell carcinoma (p<0.001and p=0.003, respectively). In the SCLC cases, the intercellular distance was less than in the adenocarcinoma and large cell carcinoma cases.
For CL-16-binding tumours, a significant difference was found only in the distenca between the negative tumour cells (p=0.034). The largest distance was seen for the pT1 tumours; the distance was less for the pT2 tumours than for the pT3 tumours. The structural characteristics did not correlate with the lymph node status. The proportion of untasined tumor cells was 25-40% lower in the squamous cell carcinoma and small cell carcinoma cases than for the other two histological types (p=0.04). The mean distances between the tomour cells and the intensely or moderately stained tumour cells were largest in the large cell carcinoma cases. As compared with the other types, the mean distance was higher in the adenocarcinoma cases, while for all three parameters the shortest distances were found in the squamous cell carcinoma and small cell carinoma cases (p<0.001, p<0.001 and p=0.012). The numbers of cells in the tumor cell clusters and in the clusters formed by the moderately and the intensely stained tumour cells were higher in squamous cell carcinoma and SCLC, while the group of cells in the adenocarcinoma and large cell carcinoma within NSCLC on average contained fewer cells (p=0.007, p=0.009 and p=0.012).
For HBL-expressing tumours, none of the various parameters exhibited a significant correlation with either the pT or the pN classification. The proportion on unstained tumour cells was lowest in sqamous cell carcinoma, where the proportion of inetnively stained cells was highest. The situation was just the opposite in large cell carcinoma. The mean distance between the tumour cells was highest in large cell carcinoma, and least in squamous cell carcinoma (p<0.001). The tendency was similar for the mean distances between the moderately and the intensely stained tumour cells (p<0.001 and p=0.001), where the mean distance of the macrocellular carcinoma cells exceed those for the adenocarcinoma and squamous cell carcinoma cells. The number of tumour cells per cluster was ~30% lower in large cell carcinoma than in squamous cell carcinoma or adenocarcinoma (p=0.006). For the clusters formed by the moderately or the intensely stained cells too, the numbers of cells were considerably lower for the patients with macrocellular carcinoma (p<0.001 and p<0.001). The highest entropy was observed in the adenocarcinoma cases (p<0.001).
For HA-binding tumours, no correlation was detected between the pT classification an the results of the syntactic structure analysis. The distance betwen the moderately stained tumour cells and the lymphocytes present in the tumour tissue was significantly larger in stage pN1 tnhan in the stage pN0 or pN2 (p=0.003). No correlation was found between other parameters and the pN. The distance between the tumour cells was ~20% greater in the macrocellolar carcinoma as in squamous cell carcinoma or adenocarcinoma (p=0.001). Corresponding differences were found for the mean diatances between the moderately and the intensely stained tumour cells (p=0.003 and p=0.002). The number of cells in the clusters of moderately and intensely stained cells were highest in squamous cell carcinoma, and progressively lower in adenocarcinoma and large cell carcinoma (p=0.002 and p=0.001). Here too, the entropy

was largest in the adenocarcinoma cases, a little lower in large cell carcinoma, and cinseiderably smaller in squamous cell carcinoma (p=0.006).
For HA-binding tumours in a Ca^{++}-containing medium, neither the pT nor the pN classification correlated with the syntactic structure analysis results. The mean distance between the tumour cells was least in squamous cell carcinoma, and greatest in macrocellular carcinoma (p=0.002). A similar tendency was observed for the distance between the moderately stained cells, where the difference was ~4 μm (p<0.001). This may be connected with the fact that the mean diameter of the clusters of moderately stained cells is greatest is squamous cell carcinoma (34.18 μm) and least in large cell carcinoma (16.75 μm) (p=0.027)

3.2.4. Correlation of the survival and the results of the syntactic structure analysis

Table 1 presents the parameters wich displayed a significant correlation with the prognosis. It is noteworthy that in 5 cases the survival improved with increase in the diameter of the tumour cell clusters. An improved survival was mainly observed for the HA-binding tumours, where increase in the number of cells in the clusters foremed by unstained tumour cells was accompanied by a better prognosis (RR: 0.949). The closest correlation with the survival was exhibited by the diamaeter of the intensely stained tumor cell clusters (p<0.001).

	Relative risk of death	95% CI			p
Galectin-3-BC					
Distance of negative tumor cells	0.9872	0.9757	-	0.9988	0.031
Diameter of negative tumour cell clusters	0.9923	0.9848	-	0.9999	0.046
Galectin-3-EX					
Diameter of intensely stained tumour cell clusters	0.9925	0.9866	-	0.9984	0.013
Hialuronic acdid-BC					
Diameter of negative tumour cell clusters	0.9906	0.9831	-	1.0077	0.015
Diameter of moderately stained tumour cell clusters	0.9845	0.9698	-	1.0198	0.043
Hialuronic acid-BC in Ca^{++}-containing medium					
Distance of lymphocytes	0.9903	0.9829	-	0.9978	0.011
Distance of neighbouring negative tumour cells and lymphocytes	0.9687	0.9446	-	0.9935	0.014
Number of negative tumour cells per cluster	0.9492	0.9045	-	0.9960	0.034
Diameter of intensely stained tumour cell clusters	0.9731	0.9584	-	0.9882	0.000

Table 1. Correlation of the results of syntactic structure analysis with the survival in lung cancer (only the significant differences are listed in the Table)

Table 2 reveals how the various parameters influence the prognosis. One of the most coherent results is furnished by the mean distance between the unstained tumour cells. In 6 of the 8 tests, an impovement in the prognosis was seen if the distance between the cells increased. For the Ga-3-binding tumours, this correlation was statistically significant. it is inetersting that, for the Gal-1-binding tumours, increase in the distance between the cells improved the survival indepently of the staining, while in the case of HA+Ca^{++} the prognosis worsened with increase in the cell distance.

In 5 examination series, increase in the mean number of tumour cells per cluster improved the survival. If the mean number uf unstained cells per cluster increased, the survival was poorer. However, for the only significant correlation, it was observed that the prognosis was improved by an increasing number of cells. For the moderately stained cells, the correlation between the survival and the number of cells is variable (thogh not significant in even a single case); for the intensely stained cells, however, it was observed in 6 tests that an increase in the number of cells was accompanied by a better survival. The prognostic role of the mean diameter of the clusters acted in virtually only one direction: as the mean diameter increased, the chance of survival decreased, though the significant nature of the correlation could not be demonstrated in even one case. Just the opposite was experienced for the cell clusters differentiated according to the staining. It was observed in a total of 5 cases that increase in a mean diameters of the differently stained cell groups was associated with a significantly better prognosis. No significant difference was observed in the case of the entropy, but a lower entropy was most often accompanied by a poorer survival.

For those parameters where a significant correlation was found with the survival, a detailed study was made of whether it is possible to form groups between which a statistically significant survival can be demonstrated, and which may therefore serve as a direct prognostic factors. As concerns the 9 possible structural properties, such groups could be formed in 4 cases (Table 3).

	Gal-1-BC	Gal-3-BC	Gal-1 EX	Gal-3 EX	CL-16	HBL	HS	HS+Ca^{++}
Proportion of negative tumour cells	↓	↓	↑	↑	↑	↓	↑	↓
Proportion of intensely positive tumour cells	↑	↓	↓	↑	↑	↓	↓	↑
Distance of tumour cells	↓	↓	↓	↑	↓	↓	↑	↑
Distance of negative tumour cells	↑	↑↑	↓	↑	↑	↑	↑	↓
Distance of moderately positive tumour cells	↑	↑	↑	↓	↑	↑	↓	↓
Distance of intensely positive tumour cells	↑	↑	↓	↑	↑	↓	↓	↓
Distance of lymphocytes	↓	↓	↑	↓	↑	↑	↓	↑↑
Distance of neighbouring negative tumour cells and lymphocytes	↑	↑	↑	↑	↓	↑	↓	↑↑
Distance of neighbouring moderately postive tumour cells and lymphocytes	↑	↑	↑	↓	↑	↓	↓	↑
Distance of neighbouring intensely positive tumour cells and lymphocytes	↑	↑	↓	↑	↓	↓	↑	↓
Number of tumour cells per cluster	↑	↑	↓	↑	↓	↑	↑	↓
Diameter of tumour cell clusters	↓	↓	↓	↑	↓	↓	↓	↓
Number of negative tumour cells per cluster	↓	↓	↓	↑	↓	↑	↓	↑↑
Diameter of negative tumour cell clusters	↑	↑↑	↑	↓	↓	↑	↑↑	↑
Number of moderately postive tumour cells per cluster	↓	↓	↑	↓	↑	↑	↑	↓
Diameter of moderately positive tumour cell clusters	↓	↓	↑	↓	↓	↑	↑↑	↑
Number of intensely positive tumour cells per cluster	↑	↑	↑	↓	↑	↓	↑	↑
Diameter of intensely positive tumour cell clusters	↓	↓	↑	↑↑	↑	↑	↓	↑↑
Entropy	↑	↑	↓	↑	↑	↓	↑	↑

Table 2. Connection between the parameters of the syntactic structure analysis and the prognosis in operated lung cancer cases (in the event of an increasing value of the parameter ↑ = better survival, ↑ ↑ = significant better survival, and ↓ = poorer survival)

	Median survival (months)	p
Galectin-3-BC		
Distance of negative tumour cells		
<=27 μm	24	0.016
>27 μm	46	
Galectin-3-EX		
Diameter of intensely positive tumour cell clusters		
<=56 μm	35	0.020
>56 μm	58	
Hialuronic acid-BC		
Diameter of negative tumour cell clusters		
<=56 μm	26	0.007
>56 μm	58	
Hialuronic acid-BC in Ca^{++}-containing medium		
Diameter of intensely positive tumour cell clusters		
<=46 μm	44	0.034
>46 μm	73	

Table 3. Parameters obtained from the syntactic structure analysis, wich may be used as direct prognostic factors

3.3. Discussion

Our investigation demonstrated that the lectin-binding and lectin expressing abilities of malignant lung cancers do not correlate with the pTN stage. Our results reveal that the survival of patients with Gal-3-binding tumours is significantly poorer than that of the negative cases. The multivariate survival study indicated the survival likewise lower in the event of gal-3-EX. Gal-3 plays a role at several points in the progression of tumours. It promotes the angiogenesis of tumour tissue (14); it serves as a substrate in the synthesis of matrix metalloproteinase-2 and -9 (15); it inhibits apoptosis (8); and the Gal-3 molecules to be found in the endothelial cells play a leading role in the binding of tumour cells (4).
The great variety of its functions may explain the paradox phenomenon that the Gal-3-EX and Gal-3-BC tend to increase in cases that are more advanced according to the T and N classification, *i.e.*cases with a poorer prognosis. Gal-3 presumably contributes to tumour progression by exerting effects at other points of attack. We have demonstrated that there is not a close correlation between the angiogenesis of tumours and the pT and pN classification; thus, the angiogenetic effec presumably contributes to tumour progression without appreciably influencing either the local growth or the lymphogenic metastasis formation (see section 4).

Our results also reveal that the Gal-1-EX and Gal-1-BC of tumour cells are accompanied by a poorer survival, whereas in the event of more advanced pT and pN classifications the proportion of positively stained cases tends to rise, in contrast with Gal-3.

Gal-1 plays a role in the interconnection of tumor cells and fibronectin, and the Gal-1-EX may be correlated with the invasive phenotype of the tumour cells. Gal-1 of stromal origin contributes to the weakening of the antitumoural immune response by inducing apoptosis of the activated T-lymphocytes (16). The interconnection of Gal-1 and the protein H-Ras(12V) that plays an important role in signal transfer is a precondition for the binding of the protein to the cell membrane and ist activation (17).

The results of our study of the hyaluronic acid-BC are apparently contradictory. Whereas the survival of the negative cases was slightly better in the Ca^{++}-free medium, the hyaluronic acid-binding cases were observed to survive longer on staining with the HA+Ca^{++} medium. A number of investigations have proved that a high HA content of the tumor stroma is an unfavourable prognostic factors: the migration of the tumor cells may be facilitated by their HA-binding receptors (CD44, RHAMM)(18, 19). This may explain why the survival is better (even if not significantly) in the case of tumours that do not bind HA. HA may interact with numerous cell-surfaces molecules. The activation of the hyalectans, wich belong among the lectins of type C and which occur, among others, on the fibroblasts, is Ca^{++}-dependent (20). We presume thet, in an environment containing Ca^{++}, HA binds to different cell-surface receptors from those to which it binds in a Ca^{++}-free medium.

We were unable tho demostrate a significant correlation between the CL-16-BC of the tumour cells, the HBL-EX and the survival.

For most of the examined lectins, considerably lower binding capacities and expressions were observed for the cells of SCLC than for those of NSCLC. We assume that the different biological behaviour of SCLC (rapid spread, early metastatization and chemosensitivity) may be connected with these characteristics, but the mechanism on wich this is based has not yet beeen clarified.

Our studises did not confirm the existence of a correlation between the presence of cell-surface lectins or lectin-binding molecules and the traditional prognostic factors. This permits a more precise assessment of the probability of survival estimated on the basis of the pTNM system.

In the course of the quantitative lectin histochemical studies, we characterized the dedifferentation accompanied by breakdown of the tissue structure with the aid of syntactic structure analysis.

For a number of lectins, it was experienced that the distance between the tumor cells generally increased as the pT stage progressed. In most tests, the intercellular distance was smaller for the pT1 tumours than for the pT2 tumours, wich corresponds to the difference between the two stages: the pT2 tumors are larger or spread to involve more of their environment (*e.g.* the visceral pleura). For Gal-1-binding and CL-16-binding-positive cells, the distance between the tumour cells is greater in stage pT2 than for pT3 tumour. One explanation of this may be that the pT3 tumours may be relatively smaller, but centrally situated, and their invasivity may be less tha that of pT2 tuomours. Increase in the intercellular distance probably depends on a decrease in the intercellular connections and an enhanced migratory ability.

When we investigated the changes in the distances betwen the moderately and the intensely stained cells separately, we observed that, mainly in the latter case, the mean distance increases as the pT stage processes. In the case of the Gal-1-BC, the differenca was significant. Gal-1 inhibits the intercellular adhesion (21), and promotes the binding of tumour cells to fibronectin (22) and hence the migration of the cells. A similar role may be played by Gal-3, which participates in the cell/fibronectin and cell laminin connection, thereby promoting the migration of tumour cells in the extracellular matrix.

The diameter of the tumor cell clusters and the number of cells forming the clusters do not exhibit a coherent correlation with the pT stage. Only for the Gal-1-expressing tumours, and within these especially for the moderately stained tumour cells, did we observe that the number of cells decreases as the pT stage progresses. As the clusters are groups composed of cells situated within a given distance, increase in the number of cells is an indirect indication of the stronger intercellular adhesion of the cells and/or their lower degree of migration.
Comparison of the results with the pN classification did not reveal a statistically significant correlation. Merely for a total of two study results did we observe that the structure analysis findings gradually changed with increase in the pN classification. In most of the examination series, not even a tendency could be detected as the pN stage change.
The above findings demostrated that the results of the syntactic structure analysis and the disintegration of the tissue structure do not correlate directly with the pTN stage.
For the NSCLC cases, particularly for the more undifferentiated large cell carcinoma cases, many study results are indicative of disorganisation of the regulaar tissue structure: an increased distance between the tumour cells, a decrease in the diameter of the tumour cell clusters, and a decrease in the number of cells forming the clusters. For the squamous cell carcinomas, these parameters demonstrated opposite results; adenocarcinoma occupies an intermediate situation. The entropy value is an exception; in most examination, this is highest in the adenocarcinoma cases; joint measurements of structure and staining therefore indicated that these are the most disorganized tumours.
It is characteristic of the values measured for SCLC that the diameter of cell clusters is generally smaller than that in the NSCLC group. The decrease in the intercellular distance or the higher number of cells per cluster is appareantly in contradiction with the very considerable migratory abilty of the tumour cells. It may be presumed that the structural characteristics change during local tumour progression. At the beginning of the process, a certain concentration of the tumour cells is necessary for the substances required to initiate angiogenesis and the breakdown of the extracellular matrix to be released in sufficient quantity from the tumour cells (22, 23); after this, the migration of the tumour cells and their entry into the blood circulation may begin. The SCLC cases involved still operable patients in a relatively early stage, and we probably succeeded in performing the syntactic structure analysis in a stage when the intercellular adhesion of the tumour cells was still comparatively strong and there was no appreciable migration. This is supported by the results detailed in section 4, wich reveal that the extent the micrivascularization in the studied SCLC cases was not more significant than in the NSCLC patients. In the NSCLC group, however, the tumours were in a different stage, where the decrease in the intercellular adhesion, and also the migration, were characteristic.
It may be concluded from the survival results that the diameter of the tumour clusters is most closely connected to the survival: the larger diameter of cell group, the better the prognosis. The greater cluster diameter means that the distance of tumour cells from one another varies within certain limits, and the tumorous progresion is in the phase of „stronger adhesion – weaker migration”. On the basis of our results, the construction of concrete prognostic categories too became possible.

4. PROGNOSTIC SIGNIFICANCE OF MICROVASCULARIZATION IN LUNG CANCER

4.1. Patients and methods

Histological material from 450 lung cancer patients who were operated radically between 1 January 1990 and 31 December 1995 was processed. 233 of the patients were operated on in the Thoracic Surgery Unit at the Thoraxklinik in Heidelberg (Germany), and the remaining 217 at the Department of Surgery of the University of Szeged. The postoperative histological examinations revealed the following cell type distribution: squamous cell carcinoma 201 (44.7%), adenocarcinoma 166 (36.9%), large cell carcinoma 65 (14.4%), small cell carcinoma 18 (4%), and the following stage distribution: 57 (12.7%) I/A, 163 (36.2%) I/B, 6 (1.3%) II/A, 87 (19.3%) II/B, 129 (28.7%) III/A, 5 (1.1%) III/B, 3 (0.7%) IV. No statistically significant difference ($p>0.05$) was seen when analyzing these data for the two different centres with exception of age.

Histological sections of 4-5 μm thickness were prepared from formalin-fixed, paraffin-embedded tissues, wich were removed from the peripherial part of the tumour. Following predigestion with trypsin, immunohistochemical staining was performed with commercially available antibody against factor VIII-associated antigen (Biogenex, San Ramon, CA). Streptavidin conjugated with alkaline phosphatase (Biogenex, San Ramon, CA) was used for labelling. Smooth counterstaining („kernechtrot") was applied to label the nuclei of the tumour cells. Both positive and negative controls were performed in staining slides with known vascular density, and by omission of the primary antibody.

Tumour vessels were defined as visible stained blood vessels located in the tumourous tissue. For measurement purposes, four areas with „normal" vascularization and two areas with markedly enhanced vascularization ("hotspots") were interactively selected and subject for digitalization, morphometric measurements based on stereological procedures and syntactic structure analysis.

The selected areas were digitalized with a colour CCD camera (JVC TK1070), at a magnification of 10x, with a resolution of 512x512 pixels. Self-made image-analysing software was used, based on the commercial Digital Image Analysing System (DIAS, Towersoft, Berlin, Germany).

From a morphometric study of selected vessels, we determined the volume fraction (Vv, the calculated volume of the vessels/the calculated volume of the tumour tissue), wich characterizes the vascular density of the given tissue; we also determined the surface fraction (Sv, the calculated surface area of the vessels/the calculated volume of the tumour tissue), wich is indicative of the intensity of the tissue oxigen supply. Of the absolute values, the smallest vessel diameter, the average vessel circumference, the vascular area and the vessel count per visual fields were measured. By means of syntactic structure analysis, we determined the distribution of the tumour cells in the vicinity of the nearest neighbouring vessel. For purposes of syntactic structure analysis, the tumour cell density (cell count per μm^2) was determined in concentric circles differing by 20 μm in radius around the vessels.

The data were subjected to statistical processing with the chi-square test and the ANOVA. The Kaplan-Meier method and Cox regression analysis were used for survival rate calculation; in the former method, the level of significance was established with a log-rank method.

4.2. Results

4.2.1. Morphometric results

4.2.1.1 On the basis of the N status

The vascularization parameters did not display a clear-cut correlation with the lymph node metastases at the various levels. The lowest vascular diameter, the average vascular circumference and the vascular area were larger for the N0 tumours than for the hilar or mediastinal lymph node metastases. In all lymph node classifications, the lowest vascular diameter was ~ 18-19 um, and the difference between the largest and smallest mean values of the average vascular surface area was similarly small (~ 8 μm). The relative data (i.e. the volume fraction an the surface fraction) were large for the N2 metastases than for the lymph node-negative cases, though the difference was only 0.3% and 0.1%, respectively. The vascular characteristics of the N1 tumours were smaller than those not only for the N2 tumours, but also for the N0 tumours. In spite of the differences observed above, the N0, N1 and N2 cases did not differ significantly from one another.

4.2.1.2 On the basis of the T status

With the exception of the number of vessels per visual field, each of the studied data was larger for the T2 tumours than for the the T1 tumours, and even exceeded those for the T3 tumours. The average values of the absolute vascularization parameters (lowest vascular diameter, average vascular circumference, vascular surface area, and the number of vessels per visual field) differed more from one another than in the various N classifications, but the differences was not significant. There was a larger difference between the group for the surface fraction (1.4%) and volumen fraction (0.4%), the former difference proving significant (p=0.022).

4.2.1.3 On the basis of the cell type

Within the NSCLCs, no essential difference was observed between the various histological types as regards the vascular morphological parameters. In the SCLC cases, most of the parameters (surface and volume fractions, average vessel circumference and vascular surface area) were larger than in the NSCLC cases, but the differences were not significant.

4.2.2. Results of syntactic structure analysis

4.2.2.1 On the basis of the N status

In the more advanced N classifications, the cell density was found to increase as the distance from vessel increased. A peak in the cell density was observed in the interval 40-60 μm, without significant difference.

4.2.2.2. On the basis of the T status

The density of tumour cells in the regions close to the vessels were largest for the T2 tumours, and somewhat smaller for the T4. The cell density was significantly lower in all regions for the T1 and T3 tumours, particularly in the intervals 20-40 μm and 40-60 μm, but the difference was not significant.

4.2.2.3. On the basis of the cell type

As regard the different histological types, the adenocarcinomas displayed the highest cell density. The cell density was similar for the squamous cell carcinomas and large cell carcinomas. The cell density for the small cell lung carcinomas was lower in every distance interval than for the other histological types. The highest cell density was observed in the interval 40-60 μm for the NSCLC cases, and in the interval 20-40 μm for the small cell lung cancer.

4.2.3. Survival results

By means of multivariate analysis, possible correlations were examined between the survival and the results to the TNM classification, the tumour volume, the morphometric parameters and the syntactic structure analysis. When all the cases were taken into consideration, the prognosis was impaired most by the appaerance of lymph node metastases ($p<0.001$). The histological type influenced the survival strongly ($p=0.002$). The survival rate was decreased significantly by the tumour cell density in the interval 0-20 μm ($p=0,02$ RR:1.05). In contrast, the increase in the cell density in the interval 20-40 μm was accompanied by a better prognosis ($p=0.046$, RR:0.93). No difference was observed as concerns the various morphometric data. A separate study was made of the parameters in the NSCLC group, where the appearance of lymph node metastases likewise meant the strongest prognostic factor ($p<0.001$). Similarly as in the above group, the survival rate was worsened by increases in the proportion of tumour cells in the interval 0-20 μm ($p=0,023$, RR:1.05), and the decreasing the cell density in the interval 20-40 μm improves the prognosis ($p=0.049$, RR: 0.93). The numerical density of tumour cell in the interval 0-20 μm and 20-40 μm was demonstrated a strong positive correlation ($r=0.963$, $p<0.001$).
If the tumour cell density within 20 μm from the vessels does not exceed 8 cells/μm^2, the survival is significantly better than at higher cell densities ($p=0.048$).

4.3. Discussion

Our studies had the aims of comparisons of the results of the stereological morphometric measurements and of syntactic structure analysis in the individual groups of classical prognostic factors (pT, pN and histological type), and of seeking correlations between the parameters characterizing the vascularization and the survival.
Study of the microvascularization necessitates quantitative characteristics, wich provide information on the degree of vascularization. The most frequently used feature is the number of microvessels per visual field. In a number of publications, an increase in the number of microvessels in lung cancer is regarded as a negative prognostic factor (6, 7). Nevertheless, it was calculated by Mattern et al., who processed the data on 87 patients with squamous cell carcinoma, that there is no correlation between the survival and the number of the microvessels (20). In our multivariate analysis, we could likewise not confirm a correlation between the survival and the number of microvessels either in the overall patients or in the NSCLC group.
Accordingly, besides the number of vessels, we measured various other morphometric parameters: the smallest vascular diameter, the average vascular circumference and average vascular surface area from the absolute vascular characteristics; and the surface area and volume of the microvessels and their ratios to the volume of the tumour (Sv and Vv) from among the relative data.
As regards the T status, we experienced that T2 tumours are more vascularized than T1 tumours, and the vascular supply of T4 tumours is better developed than that of T3 tumours. It is interesting that the degree of vascularization of T2 tumours is greater than that of T3

tumours. The value of Sv was significantly higher in the T2 cases than in the T1 and T3 cases. The number of vessels per visual field was similar in the three groups, whereas the average vascular circumference and vessel area were ~10% larger for the T2 tumours. It appears that the larger vascular circumference (and consequently the larger vascular area) is responsible for the significant change in Sv.

The density of tumour cells in the vicinity of the nearest vessels exhibits a similar tendency to that for the morphometric parameters. The cell density is highest for the T2 and T4 tumours in every distance interval. During the angiogenetic process, the basal membranes of the existing vessels are broken down by proteolytic enzymes released from the tumour cells (e.g. matrix metalloproteinase). In the course of the proteolysis, angiogenic stimulators and inhibitors are released from the extracellular matrix, and the urokinase type plasminogen activator (uPA) is upregulated by certain angiogenetic substances (24). Presumably in consequence of the higher cell density, the angiogenetic and proteolytic substances are released in higher concentrations, and this may lead to the enhanced vascularization.

We were unable to confirm the role of enhanced microvascularization in the development of lymph node metastases. The absolute parameters were largest in the event of an N0 lymph node status; only because of a lower number of vessels per visual fields was not observed a similar difference in the stereological data. Starting from the N1 status, Sv and Vv demonstrate progressive increases, but the differences are not significant. However, their tendency reveals that the degree of vascularization may be higher in the event of more advanced node metastases. The tumour cell density displays a gradual increase corresponding to the pN status, but the difference is minimal in every distance interval, and we consider that this does not influence the lymphogenic metastasizing ability of the tumour.

The morphometric parameters demonstrated that for the other histological types, the SCLC cases exhibited an enhanced vascularization. With regard to the biological behaviour of small cell carcinomas (rapid haematogenic metastatization), this results is not surprising. In particular, the average value of surface fraction exceeds the corresponding results for the other histological groups by >10%. The average values of the vascular circumference and area and of Vv also higher. As the number of vessels per visual fields is lower than in the NSCLC cases, the higher values of Sv and Vv are clearly due to the larger size of the vessels. The cell density in the region close to the vessels was lower than in the NSCLC group. The differences between the SCLC and NSCLC groups were not significant. This too may explain the fact that in these SCLC cases the tumour is in an early stage and operable, the 5-year survival rate exceeding 20%. Within the NSCLC group, no differene was found.

Of the vascularization parameters, an increase in the density of tumour cells within a distance of 20 μm from the closest neighbouring vessel was accompanied by a poorer survival This result lends support to our view that spreading of a tumour, and hence the prognosis, is influenced jointly by the angiogenesis and by the migration and proliferation of the tumour cells. This may explain why none of the morphometric parameters characterizing angiogenesis alone correlated significantly with the survival.

Our results reveal weak correlations between the extent of vascularization and the various tumour stages; in general, the more advanced pN and pT classifications are accompanied by an elevated level of vascularization, wich is primarily manifested in the Sv and Vv data and in the density of tumour cells. The survival of the patients is affected most strongly by the appearance of lymph node metastases. Of the vascular parameters, the density of tumour cells close to the vessel has a disadvantageous influence an the fate of the patients. If the tumour cell density does not exceed 8 cells/μm^2, the survival is significantly better, and this may be utilized in the direct prognosis determination.

5. SUMMARY

By means of qualitative immunohistochemical investigation on a large number of patients, we demonstrated that the prognosis of radically operated lung cancer patients is significantly worsened by the galectin-1-expressing ability and galectin-3-binding capacity of the lung carcinoma cells. The expression and binding capacities of these galectins do not correlate with the T stage or with the appearance of lymph node metastases. In a multivariate study, of a number of prognostic parameters the galectin-3-binding capacity and the galectin-3 expression proved to be independent prognostic factors. As no correlation can be detected with the pTN classification, they presumably play a role in tumor progression in some other way (by influencing the cell-cell and cell-extracellular matrix connections). The proportion of tumours giving a positive reaction is significantly smaller in SCLC than in NSCLC cases. We consider that the difference observed in the phenotype is connected with the different biological behaviour of SCLC.

The results of studies of the tissue structure (syntactic structure analysis) do not reveal a significant correlation with either the pT or pN classification. In a number of examination series, we observed that the tumor cells and (in separate studies) the distances between the moderately and the intensely stained cells gradually grow as the pT stage progresses, though these correlations are not significant statistically. In most cases, the structure analysis results do not even exhibit a tendency to follow the changes in pN.

In the more differentiated NSCLC types (squamous cell carcinoma and adenocarcioma), the distances between the tumour cells are smaller, while the diameters of the cell clusters are larger than in the more undifferentiated large cell carcinoma. These data indicate that the adhesion of cells decreases for the undifferentiated tumours and the migration is enhanced, and this process can also be characterized quantitively.

Increase in the diameter of the tumour cell clusters proved to be a favourable prognostic factor in a various examination series. The explanation of this may be that in these cases the intercellular binding is still strong and the migration of the tumour cells is minimal. For four parameters, we could establish categories suitable for direct prognosis determination: for galectin-3-binding tumours a mean distance >27 μm between the unstained tumour cels, for galectin-3-expressing tumours a diameter >56 μm for the intensely stained tumour cell clusters, for hyaluronic acid-binding tumours a diameter >56 μm for the unstained cell clusters, and for Ca^{++}-mediated hyaluronic acid-binding tumours a diameter >46 μm for the intensely stained tumour cell clusters were all associated with a significantly better prognosis.

In a study of microvascularization on a large number of patients, we confirmed that the data obtained by morphometric and syntactic structure analysis do not display a correlation with the pTN classification or the histological type. A significant correlation was found only between the surface fraction an the pT. The densities of tumor cells situated within 20 μm of the microvessles, or in the interval between 20 and 40 μm, proved to be independent prognostic factors. In the event of a tumor cell density of fewer than 8 cells/μm^2 within 20 μm, the survival is significantly better. This parameter is not only suitable to characterize the microvascularization, but also provides a guide to the migratory ability of the cells; accordingly, the quantitative characteruzation of the progression may be more exact.

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