The role of microvascularization and growth/adhesion-regulatory lectins in the prognosis of non-small cell lung cancer in stage II

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Abstract

Objective: To investigate the role of growth/adhesion-regulatory lectins in the prognosis of the stage II non-small cell lung carcinomas (NSCLCs) via quantitative lectin histochemical examinations and measurement of microvascularization of the tumour.

Methods: In 94 radically operated lung cancer patients, stage II NSCLC was confirmed histologically (T1N1: 6, T2N1: 66, T3N0: 22). Immunohistochemical methods were applied to investigate the galectin-1, galectin-3, CL-16 and hyaluronic-acid-binding capacities of the tumours, and also the expression of galectin-1, -3 and heparin binding lectin. Sections were examined with the aid of qualitative (stained/not-stained) and syntactic structure analysis. The microvessels were detected by staining with anti-factor VIII antibodies. The findings were compared with the survival data.

Results: In the univariate survival examinations, the prognosis was poorer for the galectin-1 and -3-expressing tumours ($p = 0.014$ and $p = 0.003$) and in multivariate analysis for the galectin-3-expressing tumours ($p = 0.046$, RR: 2.026). Correlations could be demonstrated between the survival and the distance between the tumour cell for the tumours binding galectin-3 ($p = 0.039$, RR: 5.944) and expressing galectin-3 ($p = 0.041$, RR: 3.335). An elevation of the volume fraction of microvessels was a sign of a poor prognosis ($p = 0.017$, RR: 2.334), however the increase of surface fraction improves the survival ($p = 0.01$, RR: 0.956).

Conclusions: In stage II NSCLC, galectin-3 expression is indicative of a poor prognosis. In tumour expressing and binding galectin-3, the distance between the tumour cells is of prognostic significance. An increase in the microvessel volume fraction points to a poorer survival rate.

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Keywords: Lung cancer; Lectins; Microvascularization; Syntactic structure analysis

1. Introduction

Study of the biological markers of tumour growth and spread is of direct clinical importance. Revelation of the exact molecular mechanism of tumour progression allows determination of those markers that serve as a better guide to the prognosis during routine immunohistochemical examinations. The basis of our investigation was that the carbohydrate molecules that appear on the surface of the cells, and the proteins binding them, the lectins, undergo a quantitative change in the course of the change in malignancy, of the tumour [1,2], and this influences the proliferation, adhesion and migration ability of the tumour cells and angiogenesis [3–7]. The prognostic role of the lectins has been studied in connection with various tumour types, and the contradictory results indicate that further studies are required at concerns the given tumour type [8].

The importance of the microvascularization has been confirmed in a number of investigations of lung cancer [9–12], but a detailed quantitative analysis if the microvascularization and its correlation with the chance of a cure were first described by our group [10,13]. A combination of the examinations may be a basis of the development of a molecular substaging system [11]. Both the literature data and our own results indicate that this promotes a better decision regarding the prognosis within a given stage [11,13,18].

Surgical treatment is still the primary strategy for patients with non-small cell lung cancer in stage II, but prospective...
randomized studies have demonstrated that these patients may profit from adjuvant chemotherapy [14], which may prevent the development of metastases from existing tumour cellular spread. However, it cannot be established in which patients the occurrence of distant metastases must be reckoned with, and in which of them adjuvant treatment may furnish a survival benefit. Investigation of the lectins and the microvascularization in this stage may be of help in the selection of those patients in whom chemotherapy may indeed be effective, and in whom effective molecular targeted therapy may be administered.

2. Patients and methods

Processing was performed on histological material from a total of 94 patients operated on in two centres (Thoraxklinik, Heidelberg, Germany, and Dept of Surgery, Szeged, Hungary). In course of preoperative examinations, all of the patients underwent routinely bone scintigraphy and abdominal ultrasound or CT as staging examinations. When new neurological complaints or symptoms occurred, CT scan of the brain was performed. Twenty-four patients underwent staging mediastinoscopy to exclude mediastinal lymph nodes involvement. Radical surgery including mediastinal lymphadenectomy was performed in all cases, and the postoperative histology confirmed non-small cell lung cancer of stage II. Statistically significant differences were not found according to the age, gender, histology, pT, pN, type of resections and survival between the data on the patients enrolled at the two centres (Table 1), therefore the combined analysis do not influence the final results.

2.1. Lectin immunochemistry

Sections, 4—6 μm thick were prepared from the paraffin embedded tumour tissue and an immunohistochemical staining technique was performed. The lectin-binding capacities of the tumour cells were studied with the aid of labelled (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 Ca²⁺-free (HA) or contained 8 mmol/l Ca²⁺ (HA + Ca²⁺). The final dilution of all probes measured 10 μg/ml for 60 min. 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). Haematoxylin 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 lectin.

During the routine light-microscopic examinations, the sections were all evaluated by the same pathologist, who classified them as negative or positive based on 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, Germany), for which 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 (d₀) did not exceed the sum of the mean intercellular distance (m) and twice the standard deviation (SD); (d₀ < m + 2sd). The structural entropy was determined by the method of Kayser and Gabius [15], with use of the differences in distance and staining between the cells.

In the course of the syntactic structure analysis, the following parameters were determined:

1. The proportion of non-stained, moderately and intensely stained tumour cells (%).
2. The mean distance between tumour cells (μm).
3. The mean distance between non-stained, moderately and intensely stained tumour cells (μm).
4. The mean distance of lymphocytes (μm).
5. The mean distance of non-stained, moderately and intensely stained tumour cells (μm).
6. The mean number of tumour cells per clusters.
7. The mean number of non-stained, moderately and intensely stained tumour cells per clusters.
8. The mean diameter of clusters of tumour cells (μm).
9. The mean diameter of clusters of non-stained, moderately and intensely stained tumour cells (μm).
10. The structural entropy.
2.2. Immunohistochemical staining of microvessels

Histological sections of 4–5 μm thickness were prepared from formalin-fixed, paraffin-embedded tissues, which were removed from the peripheral 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). Antibody binding was detected with the use of monoclonal IgG against mouse immunoglobulin; streptavidin conjugated with alkaline phosphatase (BioGenex, San Ramon, CA) was used for labelling. Nuclear fast red counterstaining 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 10 × s, with a resolution of 512 pixels × 512 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), which 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), which is indicative of the intensity of the tissue oxygen 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 μm²) was determined in concentric circles differing by 20 μm in radius around the vessels.

The finding was compared with the survival data. The end point of the examination was the death of the patients. Statistical analyses were performed with the program SPSS 13.0, with the chi-square test, and with the independent t-test. The univariate survival study was carried out with the Kaplan–Meier method and by means Cox regression with continuous-valued parameters. Parameters exhibiting significant differences were examined in multivariate analysis with the Cox proportional hazard method.

3. Results

Of the histopathological parameters, a significant difference was displayed by the histological type where the survival of large-cell lung cancer was essentially poorer than for squamous cell cancer or adenocarcinoma (57.2 and 48

<table>
<thead>
<tr>
<th>AT 80.0</th>
<th>70.0</th>
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<tbody>
<tr>
<td>60.0</td>
<td>50.0</td>
</tr>
<tr>
<td>40.0</td>
<td>30.0</td>
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<tr>
<td>20.0</td>
<td>10.0</td>
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Table 2

Univariate survival analysis of pathological and lectinhistochemical features

<table>
<thead>
<tr>
<th>Histology</th>
<th>Median survival (months)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell cc.</td>
<td>57.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adenocc.</td>
<td>48.0</td>
<td></td>
</tr>
<tr>
<td>Large cell cc.</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>27.4</td>
<td>0.597</td>
</tr>
<tr>
<td>pT2</td>
<td>52.3</td>
<td></td>
</tr>
<tr>
<td>pT3</td>
<td>50.6</td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>50.6</td>
<td>0.871</td>
</tr>
<tr>
<td>pN1</td>
<td>50.8</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1](image.png)

Fig. 1. The survival of non-small cell lung cancer in stage II according to the galectin-1 expression and the number of the patients remaining at risk (number of positive cases = 40, number of negative cases = 54) (p = 0.014).
Vessels count/visual field 0.430 0.984 0.946—1.024

Tumour cell density

m

Tumour cell density 40—60

m

Tumour cell density 20—40

m

vascularization features

Multivariate survival analysis of pathological, lectinhistochemical and micro-

Table 4

Survival analysis according to the microvascularization parameters

<table>
<thead>
<tr>
<th>p-Value</th>
<th>Relative risk</th>
<th>95.0% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface fraction</td>
<td>0.011</td>
<td>0.962</td>
</tr>
<tr>
<td>Volume fraction</td>
<td>0.025</td>
<td>2.180</td>
</tr>
<tr>
<td>Smallest diameter</td>
<td>0.348</td>
<td>0.952</td>
</tr>
<tr>
<td>Average circumference</td>
<td>0.516</td>
<td>0.989</td>
</tr>
<tr>
<td>Average vessels area</td>
<td>0.043</td>
<td>1.001</td>
</tr>
<tr>
<td>Tumour cell density &lt;20 μm</td>
<td>0.292</td>
<td>1.065</td>
</tr>
<tr>
<td>Tumour cell density 20—40 μm</td>
<td>0.793</td>
<td>0.966</td>
</tr>
<tr>
<td>Tumour cell density 40—60 μm</td>
<td>0.872</td>
<td>1.023</td>
</tr>
<tr>
<td>Tumour cell density 60—80 μm</td>
<td>0.705</td>
<td>0.969</td>
</tr>
<tr>
<td>Tumour cell density &gt;80 μm</td>
<td>0.581</td>
<td>1.015</td>
</tr>
<tr>
<td>Vessels count/visual field</td>
<td>0.430</td>
<td>0.984</td>
</tr>
</tbody>
</table>

strongest prognostic factor, increase of which was associated with a greater risk of death. Elevation of the surface fraction was accompanied by a better prognosis, but this effect was much weaker than that for the volume fraction. Increase in the mean level of vessels area exhibited a significant difference, but the confidence value revealed that its effect on survival was insignificant (Table 3).

Multivariate survival studies were performed as concerns the histological type, the galectin-1 and -3 expression and the surface and volume fractions, all of which proved to be independent prognostic factors except of galectin-1 expression (Table 4). As regard the histology, the poor survival of the large cell lung cancer cases was responsible for the significant difference; there was no difference in survival between the squamous cell cancer and the adenocarcinoma cases. The relative risk and confidence values indicated that variation in the surface fraction had practically no influence on the survival.

In the syntactic structure analysis, two parameters were found to have strong effects on the survival. The chance of survival decreased with increase of the distance between the tumour cells that were moderately stained in the tumours expressing galectin-3 (RR: 3.31, p = 0.041), and the survival similarly worsened with increase of the distance between the galectin-3-binding tumour cells expressing galectin-3 (RR: 5.944, p = 0.039) (Table 5).

4. Discussion

The galectins that occur on the surface of the tumour cells are able to bind numerous molecules. Particularly in the case of galectin-3, a large number of molecules are known whose binding is of biological importance [5]. The galectin-3-binding ligands of laminin and collagen play roles in producing sufficient quantities of matrix metalloproteinases, and in mobilizing the tumour cells in the extracellular matrix [16]. The enhancement of angiogenesis and the development of a tumour cell-endothelial connection contribute to the emergence of haematogenous metastases beside, the local tumour spread [17]. The negative effect of galectin-3 expression on the survival in lung cancer cases was demonstrated earlier [18], and its similar prognostic role in other tumour types too is also known [19]. In clinical practice, the occurrence of distant metastases is more likely for tumour cases that express galectin-3, and in these cases there may be more justification for the neo/adjuvant chemotherapy. However, the anti-apoptotic effect of galectin-3 is known, which protects tumour cells even against cisplatin in vitro [20]. Additional investigations are called for to decide whether this effect is observed in vivo, which would demand a change of the chemotherapeutic protocol.

Quantitative study of the tissue structure lends support to the role played in the progression. An increase in the distance between tumour cells that express and bind galectin-3 directly characterizes the migration, and this demonstrates a close correlation with the reduction in the survival.

Both our own earlier findings [18] and other literature results indicate that the chance of survival worsened by the enhanced expression of galectin-1 [21]. Of the various biological effects, it should be stressed that an important
precondition for activation of the oncogen H-Ras(12V) in the signal transfer of tumour cells is the binding with galectin-1 [22].

Our studies have proved that the survival of the patients is influenced by the degree of vascularization of the tumour tissue. Although this was demonstrated by the literature data for lung cancer patients, the determination of quantitative parameters other than the number of vessels per visual field was not performed earlier [12]. Our findings have demonstrated that, from among a number of factors, the volume fraction exhibits a very strong correlation with the survival. An increase in the density of vessels leads to a higher extent of microvascularization. Angiogenesis and the extracellular migration of the tumour cells are interrelated [23]; thus, an increase in the vascular density promotes both local tumour spread and haematogenous metastasization. This investigation did not reveal a correlation between the density of tumour cells situated in the vicinity of the vessels and the prognosis, in contrast with previous reports [13]. Determination of the volume fraction may serve to indicate the probable recurrence of the tumour, and it may also be of help in an assessment of the effectiveness of chemotherapy: in parallel with a greater degree of vascularization, the local drug concentration may also be higher.

In cases with non-small cell lung cancer in stage II, the galectin-3 expression and an enhanced volume fraction appear suitable for a better determination of the prognosis as concerns the routine diagnostic procedures, and as an indicator of the need or modification of the neo/adjuvant treatment.

Acknowledgements

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References


Appendix A. Conference discussion

Dr J. Edwards (Leicester, United Kingdom): There are many studies looking at novel prognostic factors in non-small cell lung cancer, some more complex to analyze than others. Some, such as tumour grade, tumour necrosis, the proliferation index, these are very simple things to do. Where do you think the expression of galectin will sit amongst these other consistent independent prognostic factors?

Dr Szoke: I think we have to look for complex systems, for example, the work of Harpole who investigated Ki-67 and angiogenesis and other prognostic factors, and we have to make complex staging systems for the analysis of the survival.

Dr Edwards: Second, are there any novel therapies that can intervene with the expression and function of the galectin proteins that may be of use in the future?

Dr Szoke: That is a complex question because the results reveal that the patients with galectin-3 expression have a poorer prognosis. That means the patients with galectin-3 expression can be candidates for adjuvant therapy, adjuvant chemotherapy, but we know that galectin-3 inhibits cisplatin-induced damage and apoptosis in tumour cells. If its effect also in vivo is investigated and revealed, it is possible that we have to change to another treatment, for example, gemcitabine or taxanes.

Dr Van Schil (Antwerp, Belgium): Did you look at the correlation between neovascularization and growth patterns? Recent data from a study we performed together with the pathologists of our institution, have shown that quite a lot of tumours have an alveolar growth pattern without any angiogenesis, and, rather paradoxically, those tumours have the worst survival. So there doesn’t seem to be a correlation between angiogenesis and long-term survival for some tumours. Did you specifically look at the growth patterns?

Dr Szoke: We didn’t investigate the many forms of angiogenesis and the effect on tumour growth.