Prognostic significance of microvascularization in cases of operated lung cancer

Tamas Szöke, Klaus Kayser, Jan-Dirk Baumhäuser, Imre Trojan, Jozsef Furak, Laszlo Tiszlavicz, Jozsef Eller, Krisztina Boda

Department of Surgery, University of Szeged, Pécsi u.4., H-6720 Szeged, Hungary
Institute of Pathology, Charite, Humboldt University, Berlin, Germany
Institute of Pathology, University of Szeged, Hungary
Department of Medical Informatics, University of Szeged, Hungary

Received 7 November 2004; received in revised form 15 January 2005; accepted 20 January 2005; Available online 2 March 2005

Abstract

Objective: Measurement of microvascularization and determination of its prognostic significance in cases of lung cancer. Methods: Section prepared from histological material from 432 radically operated non-small cell lung cancer patients were stained with antibodies against factor VIII-associated antigen. During computer imaging, the absolute and relative parameters of the vascularization were determined, as was the density of tumour cells situated in the vicinity of the vessels. The results were compared with the TNM status, the cell type and the survival. Results: Each parameter demonstrated an enhanced vascularization in classifications T2 and T4, but only the surface fraction, the mean vascular circumference and the mean vascular area displayed a significant change. The microvascularization parameters did not differ significantly between with different N status, however, the cell density progressively increased in the areas close to the vessels in advanced pN classifications. Elevation of the tumour cell density within 20 μm distance of the vessels was accompanied by a significantly poorer survival rate. Conclusions: More advanced tumour classifications grow with enhanced vascularization. A clear-cut connection cannot be demonstrated between the vascularization and appearance of lymph node metastases. The density of tumour cells measured in the direct vicinity of vessels is an important prognostic factor. © 2005 Elsevier B.V. All rights reserved.

Keywords: Lung cancer; Angiogenesis; Survival; Syntactic structure analysis

1. Introduction

In cases of lung carcinoma, the prognosis is nowadays routinely determined on the basis of the TNM system. At the same time, even for the T1N0M0 classification, the 5-year survival rate is only around 70%, i.e. even after radical tumour removal the disease recurs in every third patient [1]. Numerous histopathological or molecular genetics examinations have been performed, with the aim of studying the factors that influence the spreading of the tumour, and hence of attaining a better determination of the prognosis and of achieving new treatment procedures [2].

From the aspect of growth and spreading of the tumour, an important process is that of formation of new vessel, i.e. angiogenesis. The growth of tumours larger than 2 mm demands new capillaries, with structures differing from that of the normal vessels: they are irregular, twisted, with no smooth muscle elements, and with inadequate endothelial lining and basement membrane, which are constantly broken down by the tumour cells [3]. The prognostic value of angiogenesis was first described by Macchiarini for non-small cell lung carcinoma (NSCLC) [4], and that finding has subsequently been confirmed by other authors [5-8]. A number of studies have confirmed the prognostic value of angiogenesis in human carcinomas of the breast [9], stomach [10], large bowel [11] and ovarium [12]. However, a correlation cannot be demonstrated between the vascularization of the tumour and the prognosis in all cases [13].

The correlation between the metastatization of the tumour and angiogenesis is similarly contradictory: a correlation was reported between the appearance of lymph node metastases and the vascularization of lung carcinoma in one study [14], but others did not confirm this observation [10,15].

The aims of our retrospective study were to provide a qualitative characterization of the vascularization of lung tumours, and to seek correlations between the vascularization of radically operated lung carcinomas and the stage of the tumour or the prognosis.
2. Patients and methods

Paraffin-embedded histological material from 432 non-small cell lung cancer patients who were operated radically between 1st January 1990 and 31st December 1995 was processed. Two hundred and sixty two patients were operated on in the Thoracic Surgery Unit at the Thoraxklinik in Heidelberg (Germany), and the remaining 206 at the Department of Surgery of the University of Szeged. All of patients agreed to the subsequent scientific process of their histological material at time of the operation. Table 1 displays an overview of the clinical data including cell types and tumour stages. No statistically significant difference \( P > 0.05 \) was seen when analyzing these data for the two different centres with exception of age (Table 1). The median follow-up period was calculated from the date of surgery to 48 months (range: 2–122 months).

Histological sections of 4-5 \( \mu \text{m} \) 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). 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 \( 10 \times \), with a resolution of \( 512 \times 512 \) pixels. Self-made image-analysing software was used, based on the commercial Digital Image Analysing System (DIAS, University of Jena, Germany).

From a morphometric study of selected vessels, we determined the volume fraction (\( V_v \), 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 (\( S_v \), 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 (the theoretical background and practical details of which were reported earlier [5]), 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 \( \mu \text{m}^2 \)) was determined in concentric circles differing by 20 \( \mu \text{m} \) in radius around the vessels.

The data were subjected to statistical processing with the \( \chi^2 \) 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. The SPSS 11.0 (SPSS Corp., Chicago, IL) program was utilized for statistical processing.

3. Results

3.1. Morphometric results

3.1.1. 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 \( \sim 18-19 \mu \text{m} \), and the difference between the largest and smallest mean values of the average vascular circumference was similarly small (\( \sim 4 \) \( \mu \text{m} \)) (Table 2).

The relative data (i.e. the volume fraction and the surface fraction) were large for the N2 metastases than for
the lymph node-negative cases, though the difference was only 0.3 and 0.17%, respectively. The vascular characteristics of the N1 tumours were smaller than those not only for the N2 tumours, but also for the N0 tumours; an exception here was the number of vessels per visual field.

Because of the small number of elements (N = 2), the data on the N3 cases could not be evaluated, and accordingly these were not taken into consideration in the significance calculations. In spite of the differences observed above, the N0, N1 and N2 cases did not differ significantly from one another.

3.1.2. 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 T1 tumours, and even exceeded those for the T3 tumours. As concerns the absolute vascularization parameters (the lowest vascular diameter, the mean vascular circumference and area, and the number of vessels per field of view) differ from one another more clearly than in the various N classifications; the mean vascular circumference and vascular area differ significantly.

There was a larger difference between the group for the surface fraction (~18%) and volume fraction (~8%), the former difference proving significant (P = 0.011).

The strongest vascularization was observed for the T4 tumours. However, because of the low number of cases (N = 4) as compared with the other groups, this observation was merely of an informative nature an (similarly as for the N3 cases) this group was omitted from the significance calculations. The average vascular diameter and surface area were not larger for the T4 tumours than for the less advanced forms, but the numbers of vessels per visual fields (and consequently the values of Sv and Vv) were considerably higher than the values for the former groups.

3.1.3. 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.

3.2. Results of syntactic structure analysis

3.2.1. 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. This was followed by a temporary decrease, but at distances > 80 μm, the cell density was significantly higher than at all smaller distances. Similarly as for the morphometric data, the N3 cases were omitted from consideration because of low number of elements (N = 2) (Table 3).

3.2.2. 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 tumours (which were omitted from the calculations because of low number of cases (N = 4)). The cell density was significantly lower in all regions for the T1 and T3 tumours, particularly in the intervals 20–40 and 40–60 μm. These results demonstrated a tendency similar to that for the morphometric parameters. Here too, the interval 40–60 μm exhibited the highest cell density as concern the regions in the vicinity of the vessels.

3.2.3. On the basis of the cell type

As regarding 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 highest cell density was observed in the interval 40–60 μm for the NSCLC cases.

The direct tumour invasion did not correlate to the degree of microvascularization and to the prognosis (Table 4).

3.3. 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
Because of low numbers, the pT4 and N3 cases were omitted from the significance calculations.

The prognosis was impaired most by the appearance of lymph node metastases ($P < 0.0001$). The survival rate was decreased significantly by the tumour volume ($P = 0.018$) and the tumour cell density in the interval 20–40 $\mu m$ ($P = 0.025$). In contrast, the increase in the cell density in the interval 20–40 $\mu m$ was accompanied by a better prognosis ($P = 0.048$). No difference was observed as concerns the various morphometric data. This indicates that an enhanced vascularization itself does not automatically mean that the spreading of the tumour will be easier. An effect is also exerted by the number of tumour cells in the vicinity of the vessels formed during angiogenesis (Table 5).

### 4. Discussion

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 or they may be new vessels produced in response to the resulting angiogenic factors, which are formed from the growth of endothelial cells of the capillaries and venules [16]. The endothelium of the new capillaries has numerous trans-endothelial channels and a fenestrated or discontinuous lining [17]. The tumour cells cause the basement membrane of the proliferating tissues 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 [18]. The importance of angiogenesis in the progression of tumour has been demonstrated by a number of investigations [8,11,12,19].

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.

The vessels were defined as structures which stained positively with anti-factor VIII antibodies. We did not investigate whether the vascular structures to be found in the tumourous area were new vessels formed in the course of angiogenesis, or were vessels already existing in the normal lung tissue, but engulfed by the tumour.

### Table 5

Multivariate analysis of survival of the radically operated non-small cell lung cancer patients according to histological type, TNM classification, quantitative vascular features and syntactic structure analysis data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Relative risk</th>
<th>$P$</th>
<th>95.0% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>0.035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous vs. Adeno</td>
<td>1.37</td>
<td>0.022</td>
<td>1.05–1.80</td>
</tr>
<tr>
<td>Squamous vs. Large Cell</td>
<td>1.42</td>
<td>0.043</td>
<td>1.01–2.80</td>
</tr>
<tr>
<td>PT</td>
<td>0.106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 vs T2</td>
<td>1.36</td>
<td>0.120</td>
<td>0.90–2.00</td>
</tr>
<tr>
<td>T1 vs T3</td>
<td>1.65</td>
<td>0.034</td>
<td>1.04–2.64</td>
</tr>
<tr>
<td>PN</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO vs N1</td>
<td>1.48</td>
<td>0.012</td>
<td>1.09–2.00</td>
</tr>
<tr>
<td>NO vs N2</td>
<td>2.31</td>
<td>&lt;0.0001</td>
<td>1.69–3.16</td>
</tr>
<tr>
<td>Volume of connective tissues</td>
<td>1.00</td>
<td>0.378</td>
<td>0.99–1.00</td>
</tr>
<tr>
<td>Sv</td>
<td>1.00</td>
<td>0.646</td>
<td>0.99–1.00</td>
</tr>
<tr>
<td>Vv</td>
<td>1.07</td>
<td>0.571</td>
<td>0.85–1.32</td>
</tr>
<tr>
<td>Smallest diameter</td>
<td>1.00</td>
<td>0.948</td>
<td>0.96–1.04</td>
</tr>
<tr>
<td>Mean circumference</td>
<td>0.99</td>
<td>0.318</td>
<td>0.98–1.00</td>
</tr>
<tr>
<td>Mean area</td>
<td>1.00</td>
<td>0.204</td>
<td>1.00–1.00</td>
</tr>
<tr>
<td>Numerical density &lt; 20 $\mu m$</td>
<td>1.05</td>
<td>0.025</td>
<td>1.01–1.09</td>
</tr>
<tr>
<td>Numerical density 20–40 $\mu m$</td>
<td>0.93</td>
<td>0.048</td>
<td>0.86–0.99</td>
</tr>
<tr>
<td>Numerical density 40–60 $\mu m$</td>
<td>1.05</td>
<td>0.108</td>
<td>0.99–1.10</td>
</tr>
<tr>
<td>Numerical density 60–80 $\mu m$</td>
<td>0.99</td>
<td>0.323</td>
<td>0.96–1.01</td>
</tr>
<tr>
<td>Numerical density &gt;80 $\mu m$</td>
<td>0.99</td>
<td>0.615</td>
<td>0.97–1.01</td>
</tr>
<tr>
<td>Vessel count</td>
<td>0.99</td>
<td>0.239</td>
<td>0.97–1.00</td>
</tr>
<tr>
<td>Volume of tumour</td>
<td>1.00</td>
<td>0.018</td>
<td>1.00–1.00</td>
</tr>
</tbody>
</table>
The degree of microvascularization is most frequently characterized by the number of microvessels per field of view. 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. [20] 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. In our multivariate analysis, we could likewise not confirm a correlation between the survival and the number of microvessels.

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. However, it must be remembered, particularly for T3 tumours, that the classification fundamentally depends not so much on the size of the tumour as on its location. Further studies are required to decide whether the vascularization of the ‘invasive’ T3 forms (chest wall, pericardium and diaphragm infiltration) is more enhanced than that of cases classified as T3 on the basis of the localization (closer than 2 cm from the bifurcation). 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 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.

Angiogenesis is only one of the factors involved in the progression and metastatization of tumours. In response to the enhanced production of certain enzymes (collagenase and matrix metalloproteinases), the stroma surrounding the tumour cells disintegrates; the tumour cells then undergo migration and subsequently are able to enter the microscopic vessels. Determination of the density of tumour cell situated in the vicinity of the microvessels is used to characterize this process. Kayser et al. have dealt in a number of publications with the questions of how the steric locations of the tumour cells and their distances from other cells and tissue structures vary in lung tumours of different histological types, and what correlations they display with the survival [21]. By means of syntactic structure analysis, they demonstrated that the proportion of tumour cell in the S phase progressively rises with decrease in the distance measured from the nearest neighbouring vessel [5].

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 angiogenic 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 [22,23], and the urokinase type plasminogen activator (uPA) is upregulated by certain angiogenic substances [24]. Presumably in consequence of the higher cell density, the angiogenic and proteolytic substances are released in higher concentrations, and this may lead to an enhanced vascularization and to more aggressive growth.

We were unable to confirm the role of enhanced microvascularization in the development of lymph node metastases. Starting from the N1 status, Sv and Vv demonstrate progressive increases, but the differences are not significant. A stronger correlation between the microvascularization and the lymph node metastases has primarily been described in cases of breast cancer [9]; for other tumours (including lung tumours) a correlation could not be detected between the microvascular count and lymphogenic metastases [8,10,11], though a close correlation was observed by Slodkowska between the lymph node status and the degree of vascularization in adenocarcinomas [14]. Syntactic structure analysis reveals that the density of the tumour cells gradually increases in accordance with the more advanced lymph node metastases, but this difference is minimal in all distance ranges. Our results lead us to believe that increase in the degree of microvascularization of lung tumours does not influence the lymphogenic metastatizing ability of these tumours.

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. Kayser et al. [5] reported that the proportion of proliferating cells within 20 μm from the vessels adjacent to the tumour is higher than in more remote areas. Kirkali proved a connection between angiogenesis and the proliferation rate [13]. These results lend 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.

Elevation of the density of tumour cell located with the 20 μm range decreases the chance of survival. A higher density of cells in the interval 20–40 μm, however, influences the survival rate in a favourable direction. This can not explain in that the tumour cells are farther from the vessels in the case of a more favourable prognosis.

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, which is primarily manifested in the Sv and Vv data and in the density of tumour cells. Of the vascular parameters, the survival of the patients is influenced significantly by the density of tumour cells lying close to the vessel. Measurement of the densities of cells within 20 μm, and between 20 and 40 μm, may possibly serve both as independent prognostic factor and as a part of a substaging system.
Acknowledgements

The authors gratefully acknowledge the financial support of the Verein zur Förderung des biologisch-technologischen Fortschritts in der Medizin e.V.

References