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The prognostic significance of vascular density in patients with squamous cell lung cancer treated surgically

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Introduction. Neo-angiogenesis is a fundamental element of tumour growth, progression and metastases. Opinions in literature differ as to the prognostic significance of vascular density (VD) in non-small cell lung cancer (NSCLC).
Material and methods we analysed VD in 40 patients treated surgically for squamous cell lung cancer (SqCLC). The FVIII in endothelial cells was visualized on formalin fixed, paraffin embedded sections, using immunohistochemical methods.
Results. The mean value of VD was 37.5±2.5. Patients high VD tumours (VD>39 vessels/mm²) achieved significantly shorter survival than those with low VD (VD≤39 vessels /mm²), p=0.0279.
Conclusion. VD should be considered in the assessment of prognostic parameters for NSCLC.

Wartość prognostyczna gęstości unaczynienia u chorych na płaskonabłonkowego raka płuc leczonych operacyjnie

Wstęp. Angiogeneza jest jednym z podstawowych procesów odpowiedzialnych za wzrost nowotworu, jego progresję i tworzenie przerzutów. W literaturze brak jest zgodności w kwestii prognostycznej wartości gęstości unaczynienia (GU) u chorych na niedrobnokomórkowego raka płuc.
Materiał i metody. GU oceniono w grupie 40 chorych na płaskonabłonkowego raka płuc, leczonych operacyjnie. FVIII został uwidoczniony w komórkach śródbłonka naczyń, na utrwalonych w formalinie skrawkach parafinowych, przy użyciu immunohistochemii.
Wyniki. Średnia GU wynosiła 37,5±2,5. Chorzy na nowotwory o dużej GU (GU>39 naczyń/mm²) przeżywali istotnie krócej niż chorzy na nowotwory o małej GU (GU≤39 naczyń /mm²), p=0,0279.
Wniosek. Parametr, jakim jest GU, powinien być brany pod uwagę przy ocenie czynników prognostycznych u chorych na niedrobnokomórkowego raka płuc.

Key words: squamous cell lung cancer, vascular density, prognostic significance
Słowa kluczowe: płaskonabłonkowy rak płuca, gęstość unaczynienia, wartość prognostyczna

Introduction

There is experimental evidence that tumour growth is dependent on angiogenesis. This process is fundamental in tumour growth, progression, and metastases [1, 2].

Intratumoral microvessel density is assumed to reflect the intensity of tumour angiogenesis [3]. In breast [4, 5], prostate [6], ovarian [7], rectal [8] and gastric carcinomas [9] high vascular density (VD) is associated with shorter survival. In NSCLC, the correlation between worse prognosis and high VD was found in most studies [10-12], however not in all [13, 14].

The analysis of vascular density in the homogenous group of SqCLC is justified, as it was found that vascular density in adenocarcinomas is higher than in SqCLC [15-17], thus possibly indicating the different biology of those two groups of NSCLC.

Recently, new anti-angiogenic drugs are being tested in clinical trials [18].

We have decided to study the vascular density in surgically treated SqCLC patients.

Material and methods

Patients

Between 1986 and 1996, 40 SqCLC patients (36 men and 4 women) were treated with radical surgery: 22 underwent

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lobectomy and 18 – pneumonectomy. Mean patient age was 58.5±1 yr.

Before surgery the patients did not receive radio- or chemotherapy. There were 20 stage I, 14 – stage II and 6 – stage IIIa patients. Each patient was followed-up for 5 years after surgery. Seventeen patients died: 4 because of loco-regional cancer recurrence and 13 due to distant metastases. Twenty-three patients are still alive without progression of SqCLC.

Methods

Fresh tumour specimens (approx. 0.5 cm^2) were delivered from the operating room, shortly after excision. All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin.

Staining procedures

Sections were cut at 4 μm, mounted on Super Frost® Plus (Menzel – Gläser, Germany) slides, and then deparaffinized and hydrated through a series of xylens and alcohols.

Endogenous peroxidase activity was blocked by H_2O_2 in methanol. Before staining, the sections were digested for 25 minutes at room temperature with 0.01% solution of trypsin (pH 7.8). Next, sections were incubated (15 minutes, room temperature) with 20% swine serum (normal), (DAKO Ltd.). Von Willebrand Factor was detected using rabbit anti-human von Willebrand factor (purified immunoglobulin fraction of rabbit antiserum), (DAKO Ltd.) diluted 1/400 in TBS (pH 7.4), (1 hour incubation, 37°C). Next, slides were incubated with secondary biotynylated antibody (DAKO Ltd.) diluted 1/800 and with avidin and biotinylated horseradish peroxidase complex (DAKO Ltd.), (30 minutes incubation at room temperature for both incubations). Diaminobenzidyine and H_2O_2 were used to visualise peroxidases (10 minutes at room temperature). In the end, slides were counterstained with hematoxylin. For negative control, rabbit serum (normal) (DAKO Ltd.) was substituted for the primary antibody.

Microvassel guantification

Vessel density (VD) was assessed at ×500 magnification (×40 objective lens and ×12.5 ocular lens; 0.145 mm^2 per field) in 7 randomly selected fields. The archival tissue sections included into analysis were of different size. In case of small samples it was impossible to find „hot spots". Therefore we decided to apply a method based on the assessment of VD in randomly selected fields for all slides. VD was expressed as a mean (of 7 values) number of vessels per 1 mm^2.

Any brown-stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumour cells and connective tissue elements, was considered as a single, countable vessel (Figure 1A). Vessel lumen was not necessary for defining a structure as a microvessel [4]. VD was assessed only in tumour tissue (Figure 1A). Vessels located near normal tissue structures were excluded from analysis (Figure 1B).

Statistical analysis

Descriptive statistics were used to determine mean values of VD and standard errors of mean (SE). In all statistical procedures, $\alpha<0.05$ was considered significant. The statistical significance of the differences between the mean values was assessed by Mann-Witney U test. Disease-specific survival (the patients whose cause of death was not malignant were treated as alive) was analysed. The probability of survival was calculated using the Kaplan – Meyer method. Univariate analysis was performed using the log-rang test. As neither median nor mean value was statistically significant, we determined an optimal cut-off point (39 vessels /mm^2) for the VD variable ('minimal' p value) by the log-rank test.

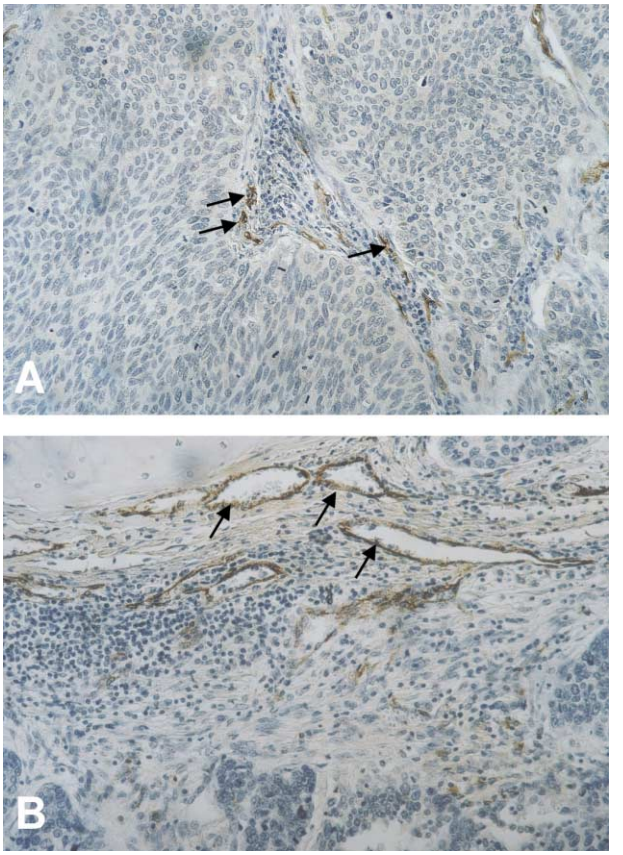

Figure 1. Vessels (indicated by arrows) in tumour tissue (A) and near normal tissue structures (B), (×160)

Results

In the analysed group of 40 SqCLC vessels were mainly located at the margins of large tumour nests (Figure 1A) and, sometimes, the microvessels penetrated into the central portions of those tumour nests. Vessels located near normal tissue structures were of larger size and sometimes were stained more strongly with anti-FVIII than tumour microvessels (Figure 1B).

VD ranged between 6.9 – 78.8 microvessels/mm^2, with a mean value of 37.5±2.5 and a median of 34.2. No differences in mean values of VD were found between the different stages of the tumours. There was no correlation between patient age and VD ($p=0.3$).

The Kaplan-Meier estimated 5-year disease-specific survival was 54%. Patients having tumours with high VD (VD>39 vessels/mm^2) achieved significantly shorter survival than those with low VD (VD≤39 vessels /mm^2), $p=0.0279$, (Figure 2, Table I).

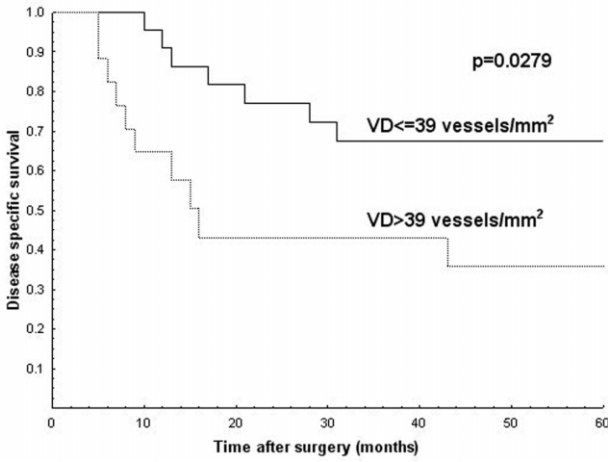

Figure 2. Correlation between vascular density (VD) and disease specific survival. Survival as a function of VD stratified using the VD≤39 vessels/mm^2 and the VD≤39 vessels/mm^2

Table I. Univariate analysis for SqCLC patients treated with surgery. Data for 5-year disease-specific survival

parameter	n	Median survival (months)	The Kaplan-Meier estimated 5-year survival (%)	(log- rank test) p value
VD				
VD≤39vessels/mm^2	23	-	67	
VD>39vessels/mm^2	17	15	36	0.0279

Discussion

The mean VD observed in our study remains within the broad range of other authors' results (Table II). Table II illustrates that there is no standardisation of VD assessment methods and hence the VD values and their prognostic significance vary in different reports. Discrepancies might be caused by different antibodies used, different methods of VD assessment, and histological heterogeneity of analysed NSCLC groups (Table II).

The choice of the optimal method for VD measurement remains a matter of discussion. Several studies have proven that anti-FVIII microvessel staining may be imprecise for several reasons: (1) FVIII is present in the lymphatic endothelium and in the platelets [20]. (2) In addition, when using anti-FVIII, newly formed vessels cannot be distinguished from older ones [20]. (3) However, the most important fact is that FVIII is not expressed in all endothelial cells, because endothelial cells of microvessels are less rich in Weibel-Palade bodies, than those of macrovessels [20]. Also, the endothelial cells of neocapilaries, when activated by cytokins (like thrombin and intrrleukins), may release their FVIII stores [20]. In our study we observed large or medium sized vessels to be stained stronger with anti-FVIII than microvessels. In other studies, [26] small vessels clearly defined in the anti-CD31 stained slides were less frequently defined in the anti-FVIII-stained slides (large or medium sized vessels were well stained with both antibodies). Besides, in a number of studies [15, 20, 21, 26, 27] the vascular density assessed using anti-FVIII

Table II. Prognostic significance of vascular density in NSCLC and methods of VD assessment. Literature data

Author, year of publication	No. of patients	Antibody	Survival	Method of VD assessment (magnification)	Mean (median) number of vessels per mm^2 of sample
Masuya et al. 2001 [17]	104	Anti-CD34	S	Average of the 3 „hot spots" (200×)	125.0± 62.8
Cagini et al. 2000 [19]	99	Anti-CD34	NS	Average of the 3 „hot spots" (250×)	50.0±17.7
Yano et al. 2000 [20]	108	Anti-FVIII Anti-CD34	NS S	Sum of the vessel count of 4 „hot spots" (200×)	11.9±5.5 19.3±6.9
Mattern et al. 1999 [14]	87*	Anti-FVIII	NS	Average of the 3 „hot spots" (250×)	16.5 (median)
Duarte et al. 1998 [21]	112	Anti-FVIII Anti-CD31	S NS	Average of the 2 „hot spots" (200×)	28.0±15.1 40.0±24.5
Matsuyama et al. 1998 [12]	101	Anti-CD34	S	Average of the 3 „hot spots" (200×)	96.1±63.6
Apolinario et al. 1997 [10]	104	Anti-CD31	NS**	Sum of the vessel count of 4 „hot spots" (400×)	106.9± 41.7
Chanrachud et al. 1997 [13]	88	Anti-FVIII	NS	"hot spot" with highest number of microvessels (200×) Average of 18 randomly selected fields (200×)	140.8 67.2
Fontanini et al. 1997 [22]	407	Anti-CD34	S	Average of the 3 „hot spots" (250×)	27.0 (median)
Fontanini et al. 1997 [11]	73	Anti-FVIII	S	"hot spot" with highest number of microvessels (250×)	19.2 (median)
Lucchi et al. 1997 [23]	227	Anti-CD-34	S	"hot spot" with highest number of microvessels (200×)	29.2±19.0
Angeletti et al. 1996 [24]	96	Anti-FVIII	S	"hot spot" with highest number of microvessels (200×)	36.7±18.9
Fontanini et al. 1995 [25]	253	Anti-FVIII	S	"hot spot" with highest number of microvessels (250×)	62.0±36.3

S – significant
NS – not significant
* SqCLC
** significant only for stage II patients

antibody was lower than when assessed with other endothelial cell markers (anti–CD31, CD-34 or PECAM). Additionally, no correlation was found between anti-FVIII and anti- CD-34 – defined VD [20] or between anti-FVIII and anti- CD-31 – defined VD [21]. These results indicate that anti-FVIII is a less sensitive marker of tumour vascularisation, which, in turn, suggests that other antibodies (anti-CD31, anti-CD34) should be recommended for VD assessment. Despite that, the prognostic significance of VD was found using both anti-FVIII and anti-CD31 or CD34 antibodies (Table II). In our study, VD assessed using anti-FVIII was also found to influence patient survival significantly.

The assessment of VD in randomly selected fields (as in our study) is rarely used by other authors [13] (Table II). However, in some studies, VD assessed using the "hot spot" method was not associated with patient survival [10, 13, 14, 20, 21]. The fact that in our study VD was a prognostic parameter might indicate that the assessment of VD in randomly selected fields might be an alternative for the "hot spot" method.

Concluding, VD should be taken into consideration during the assessment of prognostic parameters for NSCLC.

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