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Vascular endothelial growth factor expression and neovascularisation in non-small cell lung cancer☆

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Abstract

Many recent studies have demonstrated that tumour angiogenesis is a potent prognostic factor for various malignant tumours, but this has not been clearly shown in non-small cell lung carcinoma (NSCLC). The purpose of this study was to re-evaluate the prognostic value of MVD associated with VEGF in patients with NSCLC by comparing the immunohistochemical results obtained for CD34 with those obtained for vWf. Microvessel density (MVD) and the expression of vascular endothelial growth factor (VEGF) were investigated in 108 cases of NSCLC by immunohistochemistry. The correlation between von Willebrand factor (vWf) and CD34 staining for MVD was not strong, and vWf staining did not correlate with VEGF expression, but CD34 staining did. Staining for CD34 significantly correlated with survival in adenocarcinoma, distant metastasis and postoperative recurrence, but staining for vWf did not. CD34 was more sensitive and specific than vWf for staining endothelial cells associated with VEGF expression. It is suggested that research on neovascularisation should be investigated on every histological subtype or should focus on the early stages of NSCLC which are not under the influence of a variety of complications facilitating tumour neovascularisation.

Keywords: Vascular endothelial growth factor (VEGF); Angiogenesis; von Willebrand factor (vWf); CD34; Non-small cell lung cancer (NSCLC)

1. Introduction

Angiogenesis is an essential process required for the growth and metastatic ability of solid tumours [1]. Some studies demonstrated that an increase in microvascular density (MVD) was found to be closely associated with the expression of vascular endothelial growth factor (VEGF), and that MVD and VEGF expression had a prognostic value in predicting metastasis of various malignant solid tumours [2–6]. With respect to non-small cell lung cancer (NSCLC), however, few studies have investigated the association between microvascularisation and metastasis or survival. Those studies that have been published are inconclusive, as some reported a prognostic value for VEGF expression and microvascularisation [7–10], whilst others did not [11–14]. The discrepancy in results may be due to the methods used to stain the vascular endothelium. Antibodies against von Willebrand factor (vWf) were once thought to be a good marker for endothelial cells, but in-depth antibody studies have demonstrated that vWf is limited in its ability to identify endothelial cells [15–17].

2. Patients and methods

2.1. Patients

Tumour specimens from 108 patients (79 (73%) men and 29 (27%) women; mean age: 62.8 years) resected for NSCLC at Itabashi Hospital, Nihon University (Tokyo, Japan) from January 1988 to December 1992 were assessed. The patients were staged according to operating and pathological findings based on AJCC/UICC-TNM classification and stage grouping, and were classified as follows: 40 (37%) in stage I, 22 (20%) in stage II, 30 (28%) in stage IIIA, 8 (7%) in stage IIIB and 8 (7%) in stage IV. Histological classification of the tumours revealed 40 epidermoid (37%) carcinomas, 51 (47%) adenocarcinomas and 17 (16%) large cell

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carcinomas. Amongst these patients, 67 (62%) relapsed and died, whilst the remaining 41 (38%) had a median follow-up period of 31 months (range: 5–102 months). Amongst the surviving patients, 32 (30%) were disease-free for more than 5 years after surgical resection.

2.2. Immunohistochemistry

We reviewed the haematoxylin and eosin stained slides of the tumour specimens, then selected tissue blocks of the invasion edge in the tumour area for the formalin-fixed, paraffin-embedded samples of tumour tissues taken at surgical resection. Immunohistochemical studies were performed using the avidin–biotin immunoperoxidase complex technique. Staining for VEGF was performed using an anti-VEGF monoclonal antibody (MAb) (Ab-3; Calbiochem, Cambridge, UK), generated by immunising mice with a peptide from the N-terminal region of $VEGF^{165}$ and fusing it with SP2/0. Staining for vascular endothelial cells was performed on adjacent sections using an anti-vWf MAb (DAKO, Copenhagen, Denmark) and an anti-CD34 MAb (QB-END/10; Biogenesis, Sandown, USA). Briefly, formalin-fixed, paraffin-embedded 3 μm tissue sections were deparaffinised with xylene, dehydrated in ethanol and incubated with 3% hydrogen peroxidase for 5 min. After washing with phosphate-buffered saline (PBS), tissue sections were incubated in 10% normal bovine serum for 20 min, followed by an overnight incubation with anti-vWf antibody or anti-CD34 antibody at a 1:25 dilution. Biotinylated goat antimouse and antirabbit immunoglobulins were used as secondary antibodies. Peroxidase-conjugated avidin was used at a dilution of 1:500. Finally, 0.02% diaminobenzidine and 1% hydrogen peroxide in PBS was used as the substrate. Normal mouse IgG diluted to an equivalent protein concentration was used as a control in place of the primary antibody. Counterstaining was performed with haematoxylin.

2.3. Assessment of tumour vascular density

MVD as measured by vWf and CD34 immunostaining was determined using a modification of the technique described by Weidner and colleagues [18]. The four most vascularised areas which were almost at the invading margin of the tumour, were identified by light microscopy under low power magnification (×40). Individual microvessels were counted at a high-power magnification (×200; area 0.713 mm^2). The counting score was represented by the sum of the vessel count of four of these ×200 fields. Any brown-staining endothelial cells or endothelial cell clusters clearly separated from adjacent microvessels, tumour cells and connective tissue elements were considered as a single countable microvessel. Vessels with thick muscular walls or lumens larger than approximately eight red blood cells, and vessels in sclerotic areas were excluded from the count. The rationale for the exclusion of vessels with a caliber >8 red blood cells is based on studies by Van Hoef and colleagues and Querrec and colleagues [24,25]. The distribution of microvessel counts was not continuous. All tissue sections were reviewed by two independent observers without knowledge of the clinical data, and assessed as either positive or negative for VEGF expression.

2.4. Evaluation of VEGF expression

For the evaluation of VEGF expression, immunostaining was classified in two groups, corresponding to the percentage of immunoreactive cells; the cut-off point to distinguish low from high VEGF expression was 25% of positive carcinoma cells (as the mean percentage of positive cells = 25.44). The method for the sum of the percentage of positive cells was used, but the method for staining intensity [11] was not used, because the intensity of immunostaining and the staining pattern of carcinoma cells were heterogeneous, and the inflammatory cells and tissues often showed strong intensity of VEGF.

2.5. Tumour vessel invasion

The delineation of vessels with CD34 allowed the identification of neoplastic emboli as aggregates of carcinoma cells within tumour vessels as defined above. Lymphatic and blood vessels were nearly distinguishable on the basis of morphological findings such as the presence of erythrocytes.

2.6. Statistical analysis

Statview 4.0J statistical software (Abacus Conceps, Inc., Berkeley, CA, USA) was used for all analyses. The Chi-square test and Fisher's two-tailed exact test were applied to assess the correlation between immunoreactivity and clinicopathological factors. Survival curves were generalised using the Kaplan–Meier method, and prognoses were compared using the generalised Wilcoxon's analysis. A multivariate analysis was performed using the Cox proportional hazards model to investigate the independence of the risk factors.

3. Results

3.1. VEGF staining and expression

Positive staining was obtained in 49 out of 108 cases (45%) and a typical immunohistochemical staining is shown in Fig. 1. The distribution of VEGF-positivity was not continuous in the whole slide of the specimen but was judged in the area of the tumour invasive edge

where continuous staining was sometimes seen. Histologically different subtypes and different grades expressed various intensities of VEGF. Cytoplasmic immunoreactivity was present in most cases, and nuclear staining was present in some. Although most cases stained positively for both tumour cells and tumour stroma at the same time, vascular endothelium stained very weakly. As a result, VEGF expression was more intense in adenocarcinomas in comparison with other histological subtypes ($P = 0.0356$), but there was no significant correlation between VEGF expression and age, sex, differentiation, stage or vessel invasion (Table 1).

3.2. Microvessel staining and MVD

By staining with two different antibodies, we noted significant differences in the intensity of microvessel staining. It was obvious, however, that CD34 was superior to vWf in microvessel detection, as vWf stained approximately only 60% of the vessel endothelium that was stained by CD34. Representative immunohistochemical stainings are presented in Figs. 2 and 3. The correlation between MVD as determined by vWf versus CD34 staining was not significant, as indicated by a Spearman rank correlation coefficient of 0.453. The mean MVDs for all patients were as follows: vWf, 33.9 ± 15.8 and CD34, 54.9 ± 19.8. When the mean MVD was used as a cut-off point for distinguishing tumours with low and high MVDs, there was no significant correlation between MVD determined by vWf staining and various clinicopathological parameters. A significant correlation was found, however, between low and high MVDs determined by CD34 staining and sex, histological subtype and the presence of vessel invasion (Table 2).

3.3. Relationship between VEGF expression and MVD

The MVDs in the VEGF positive group were as follows: vWf, 28.3 ± 10.6 and CD34, 55.7 ± 18.3; those in the negative group were: vWf, 38.5 ± 18.2 and CD34, 54.2 ± 21.1. Although the MVDs determined by CD34 staining in the VEGF positive group were higher than those in the negative group, the reverse was seen for vWf staining (Table 3).

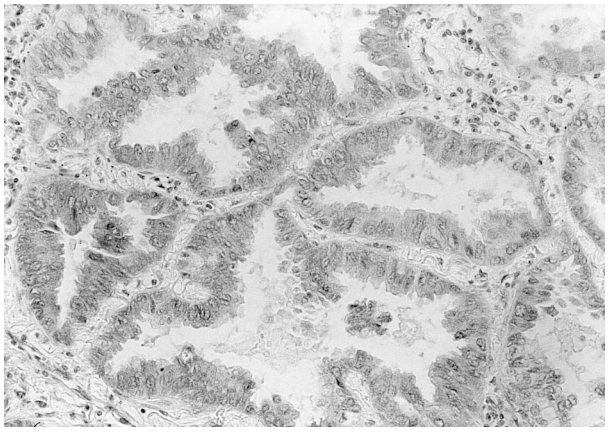

Fig. 1. Immunohistochemical staining for VEGF in well-differentiated adenocarcinoma of the lung.

3.4. Correlation between MVD and metastasis

The MVDs in the nodal metastasis positive group were as follows: vWf, 31.6 ± 13.4 and CD34, 51.0 ± 18.2; those in the negative group were: vWf, 33.6 ± 17.0 and CD34, 53.5 ± 19.4. There were no significant differences between nodal metastasis positive and negative groups, regardless of the vessel staining method. There was no significant difference between the MVDs determined by vWf staining in the distant metastasis positive group (35.1 ± 18.2) and negative group (33.5 ± 14.7), but the MVDs determined by CD34 staining were significantly higher in the distant metastasis positive group (62.4 ± 22.6) than those in the negative group (51.2 ± 17.3) ($P = 0.0122$) (Table 3).

3.5. VEGF expression and prognosis

Kaplan–Meier curves based on VEGF expression showed that there was no significant correlation between the survival time of patients with high VEGF expression and that of patients with low VEGF expression (data not shown).

Table 1
Relationship between clinicopathological factors and VEGF expression ($n = 108$)

Variables	n (%)	VEGF expression		P value
		+ n (%)	− n (%)	
Sex				
Male	79 (73)	31 (39)	48 (61)	
Female	29 (27)	18 (62)	11 (38)	NS
Subtype				
Epidermoid carcinomas	40 (37)	13 (33)	27 (68)	
Adenocarcinomas	51 (47)	29 (57)	22 (43)	0.0356
Large cell carcinomas	17 (16)	7 (41)	10 (59)	
Different grade				
Well	20 (19)	11 (55)	9 (45)	
Moderate	37 (34)	16 (43)	21 (57)	NS
Poor	34 (31)	15 (44)	19 (56)	
p-stage				
I	40 (37)	21 (53)	19 (48)	
II	22 (20)	11 (50)	11 (50)	
IIIA	30 (28)	12 (40)	18 (60)	NS
IIIB	8 (7)	2 (25)	6 (75)	
IV	8 (7)	3 (38)	5 (63)	
Vessel invasion				
Present	59 (55)	25 (42)	34 (58)	NS
Absent	49 (45)	24 (49)	25 (51)	

VEGF, vascular endothelial growth factor; NS, non significant. A P value of < 0.05 was considered significant.

Table 2
Relationship between clinicopathological factors and tumour vascularity ($n=108$)

Variables	n (%)	vWf		P value	CD34		P value
		<34 n (%)	$\geqslant 34$ n (%)		<55 n (%)	$\geqslant 55$ n (%)	
Sex							
Male	79 (73)	47 (59)	32 (41)	NS	49 (62)	30 (38)	
Female	29 (27)	16 (55)	13 (45)		10 (34)	19 (66)	0.019
Subtype							
Epidermoid carcinomas	40 (37)	27 (68)	13 (33)		33 (83)	7 (18)	
Adenocarcinomas	51 (47)	31 (61)	20 (39)	NS	22 (43)	29 (57)	0.0003
Large cell carcinomas	17 (16)	5 (29)	12 (71)		4 (24)	13 (76)	0.00007
Different grade							
Well	20 (19)	13 (65)	7 (35)		10 (50)	10 (50)	
Moderate	37 (34)	26 (70)	11 (30)	NS	24 (65)	13 (35)	NS
Poor	34 (31)	26 (76)	25 (74)		25 (74)	26 (76)	
p-stage							
I	40 (37)	26 (65)	14 (35)		27 (68)	13 (33)	
II	22 (20)	14 (64)	8 (36)		15 (68)	7 (32)	
IIIA	30 (28)	17 (57)	13 (43)	NS	10 (33)	20 (67)	NS
IIIB	8 (7)	4 (50)	4 (50)		4 (50)	4 (50)	
IV	8 (7)	2 (25)	6 (75)		3 (38)	5 (63)	
Vessel invasion							
Present	59 (55)	36 (61)	23 (39)	NS	24 (41)	35 (59)	0.0026
Absent	49 (45)	29 (59)	20 (41)		35 (71)	14 (29)	

vWf: von Willebrand factor; NS, non significant. A P value of <0.05 was considered significant.

Table 3
Relationship between MVD and tumour status or VEGF expression

Variable		MVD for vWf	P value	MVD for CD34	P value
Nodal metastasis	N_0	33.6 ± 17.0	NS	53.5 ± 19.4	NS
	N_{1-2}	31.6 ± 13.4		51.0 ± 18.2	
Distant metastasis	M_0	33.5 ± 14.7		51.2 ± 17.3	
	M_1	35.1 ± 18.2	NS	62.4 ± 22.6	0.122
VEGF expression	(+)	28.3 ± 10.6		55.7 ± 18.3	
	(−)	38.5 ± 18.2	0.0004	54.2 ± 21.1	NS

MVD, microvessel density; VEGF, vascular endothelial growth factor; NS, non significant. A P value of <0.05 was considered significant.

3.6. MVD and prognosis

For the 41 survivors, the MVDs were as follows: vWf, 31.9 ± 12.0 and CD34, 49.7 ± 15.1; those for the 67 patients who died of cancer after surgery were: vWf, 35.1 ± 17.8 and CD34, 58.0 ± 21.7. The MVDs determined by CD34 staining for the survivors were significantly lower than those for the non-survivors ($P=0.0159$), but no significance was noted for the MVDs determined by vWf staining between these two groups ($P=0.2466$). When the mean MVD was used as a cut-off point, MVDs determined by vWf staining were not prognostic; however, high MVDs determined by

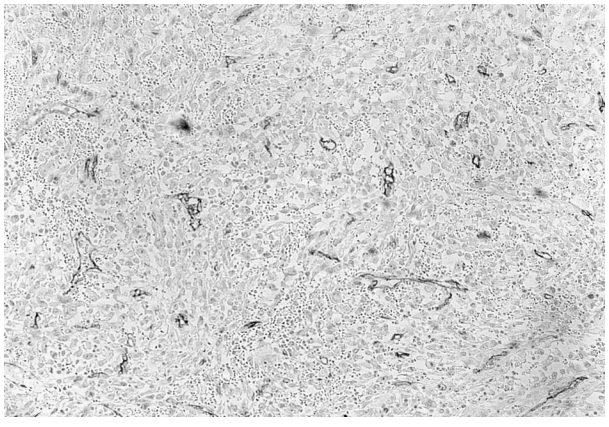

Fig. 2. Immunohistochemical staining for factor VIII-RA (vWf).

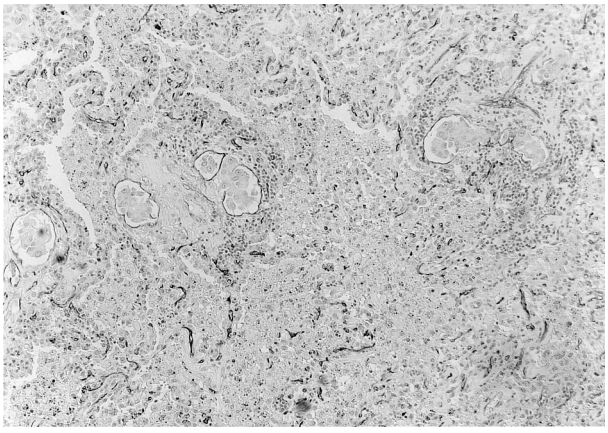

Fig. 3. Immunohistochemical staining for CD34.

Table 4
Relationship between frequency of postoperative relapse and VEGF expression in patient with NSCLC of stage I–II ($n=62$)

Postoperative behaviour	n (%)	VEGF expression		P value
		$+n$ (%)	$-n$ (%)	
Intrathoracic relapse	15 (24)	5 (33)	10 (67)	
Distant metastasis	15 (24)	10 (67)	5 (33)	NS
Relapse free	32 (52)	16 (50)	16 (50)	

VEGF, vascular endothelial growth factor; NSCLC, non-small cell lung cancer; NS, non significant. A P value of <0.05 was considered significant.

Table 5
Relationship between frequency of postoperative relapse and MVD in patient with NSCLC of stage I–II ($n=62$)

Postoperative behaviour	n (%)	MVD for vWf	P value	MVD for CD34	P value
Intrathoracic relapse	15 (24)	35.1 ± 17.6		53.3 ± 19.9	
Distant metastasis	15 (24)	32.7 ± 15.4	NS	61.6 ± 22.2	0.0190
Relapse free	32 (52)	31.9 ± 12.0		49.7 ± 15.1	

MVD, microvessel density; vWf, von Willebrand factor; NS, non significant. A P value of <0.05 was considered significant.

CD34 staining ($\geqslant 55$) significantly predicted a worse overall survival than low MVDs (<55) in cases of adenocarcinoma ($P=0.0314$) (Fig. 4).

3.7. VEGF expression and MVD in early stage patients as they relate to postoperative development of metastasis

In stage I and II cases, 15 of 62 (24%) patients developed postoperative distant metastasis during follow-up; of these, the VEGF positive group comprised 67% (10/15). The rate of VEGF positive patients in the distant metastasis group was higher than those in the intrathoracic relapse (33.3%) and the relapse-free (50%) groups (Table 4). Furthermore, the MVDs determined by CD34 staining in the postoperative distant metastasis group were higher (61.6 ± 22.2) than those in the relapse-free group (49.7 ± 15.1) ($P=0.0190$), whereas no significant difference was noted for the MVDs determined by vWf staining (Table 5). All cases that were both VEGF positive and had a high MVD determined by CD34 staining developed postoperative distant metastasis (8/8), and this rate of metastasis was significantly higher than that in the VEGF negative group and the VEGF positive, low MVD group ($P<0.0001$) (Table 6). No such correlation was noted for the MVDs determined by vWf staining (data not shown).

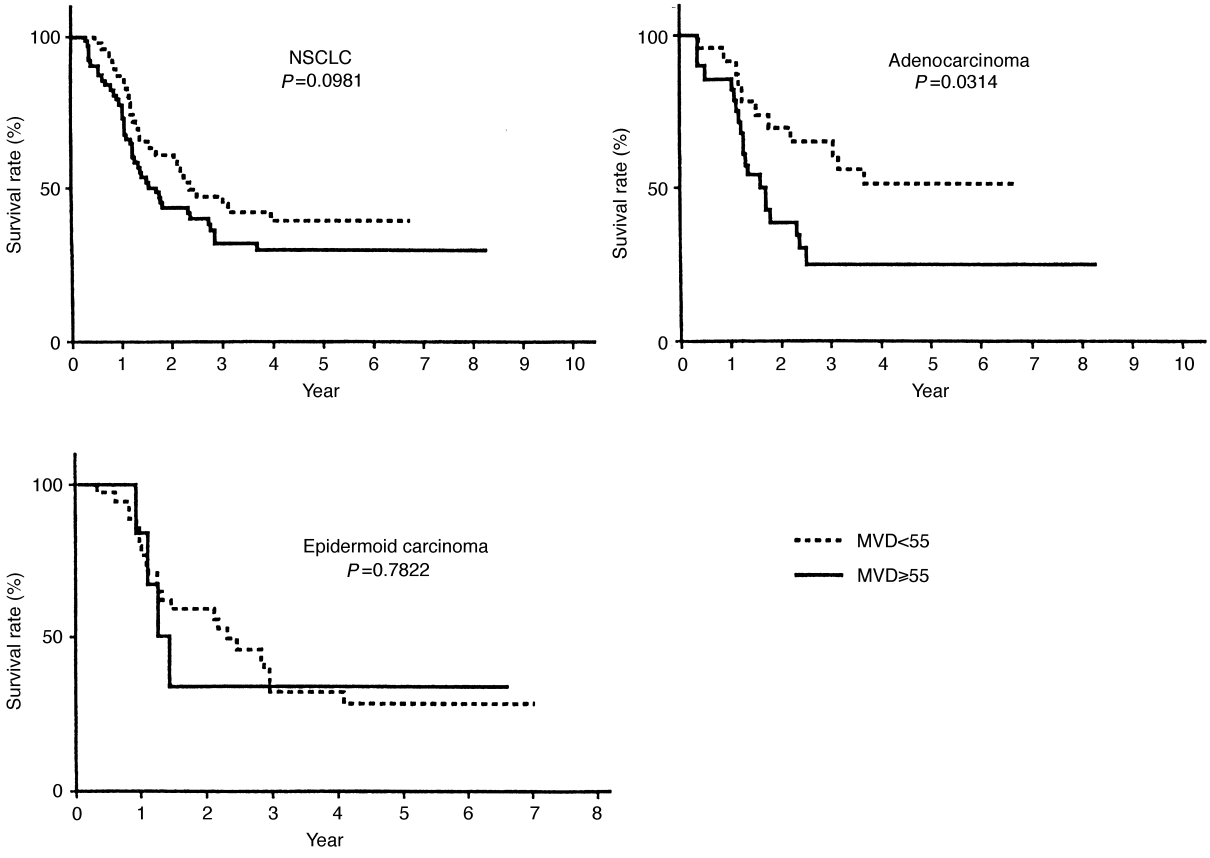

Fig. 4. Kaplan–Meier survival curves of patients with NSCLC according to microvessel density (MVD) stained for CD34.

Table 6
Relationship between neovascularisation responded to VEGF expression and postoperative distant metastasis in patients with NSCLC of stage I–II

Variable	*n* (%)	Vascularity for CD34	*n* (%)	Distant metastasis (%)	*P* value
VEGF (+)	32 (52)	High vascularity	8 (25)	8 (100)	< 0.0001
		Low vascularity	24 (75)	3 (13)	
VEGF (−)	30 (48)	High vascularity	13 (43)	2 (15)	
		Low vascularity	17 (57)	2 (12)	

VEGF, vascular endothelial growth factor. A *P* value of < 0.05 was considered significant.

3.8. Multivariate analysis

Upon multivariate analysis of all patients, the N-classification was the most significant prognostic factor for a poor survival ($P = 0.0004$). Other significant variables were vessel invasion ($P = 0.0012$), tumour grade ($P = 0.0133$) and MVD determined by CD34 staining ($P = 0.0292$). The T-classification, sex, histology, VEGF expression, and MVD determined by vWf staining were not independent prognostic factors (Table 7).

4. Discussion

Microvasculature is important in the study of cancer growth and metastasis because it is involved in the transport of various nutrients to the tumour cells [1]. Tumour microvasculature differs from that of normal tissues; for example, it consists of fragile and irregular vessels that lack the structure present in normal tissue and has increased permeability and a higher proliferation rate than that of normal endothelial cells. Furthermore, vascular endothelium can have a very different antigenic expression which depends not only on whether the tissue is malignant or benign but also on the types of vessels and organs in which the tissue lies [19].

Induction of angiogenesis is mediated by various angiogenic factors, and it has been proposed that one or more of these angiogenic growth factors may act together to induce angiogenesis and that these factors may be regulated by VEGF [20]. VEGF is a multifunctional cytokine that increases microvascular permeability and directly stimulates endothelial cell growth and angiogenesis. There are four isoforms: $VEGF^{121}$, $VEGF^{165}$, $VEGF^{189}$ and $VEGF^{206}$; of which, $VEGF^{165}$ is the most abundant in human tissues [21]. In the present study, we used a MAb specific for $VEGF^{165}$ and found no association between VEGF expression and histological grade, p-stage, TNM-classification, vessel invasion or overall survival, but noted a correlation between VEGF expression and histological subtype. These results may be explained by several factors: VEGF is induced by a variety of factors such as hypoxaemia, various cytokines, epidermal growth factor, transforming growth factor (TGF)-β1, keratinocyte growth factor and tumour necrosis factor-α to name just a few [22,23]. In lung carcinoma, such factors could be responsible for the up-regulation of VEGF in tumour cells and adjacent benign tissues because lung carcinoma is especially susceptible to infection, oppression and necrosis compared with other types of carcinomas. Therefore, it has been difficult to demonstrate an association between VEGF expression and prognosis in early stage lung cancer, which generates only small quantities of VEGF. However, VEGF expression in human malignancies has been evaluated by various methods such as immunohistochemical staining [4,8], enzyme immunoassay [2], *in situ* hybridisation [3], Northern blot analysis [6] and quantitative RT-PCR assay [7]. Regardless of the method used, there are limitations in detecting subtle differences in gene expression, evidenced by the heterogeneity of VEGF expression within even the same paraffin section

Table 7
Multivariate analysis for overall survival by Cox proportional hazards model

Variable	Categories	Hazard ratio	SEM	*P* value
Sex	Male versus female	1.697	0.30168	NS
Histology	Squamous versus non-squamous	1.067	0.28991	NS
Tumour grade	Poor versus not poor	1.931	0.26581	0.0133
T-classification	$T_{1,2}$ versus $T_{3,4}$	1.274	0.31352	NS
N-classification	N_0 versus $N_{1,2}$	2.505	0.25898	0.0004
Vessel invasion	(+) versus (−)	2.359	0.26460	0.0012
VEGF	(+) versus (−)	0.487	0.38540	NS
MVD (vWf)	< 34 versus $\geqslant 34$	1.626	0.31538	NS
MVD (CD34)	< 55 versus $\geqslant 55$	0.544	0.27899	0.0292

MVD, microvessel density; VEGF, vascular endothelial growth factor; NS, non significant; SEM, standard error of the mean. A *P* value of < 0.05 was considered significant.

or resected tissue [3,4] and by the tendency for increased VEGF expression in tissues with inflammation, necrosis and fibrosis. These results suggest that VEGF expression is not an independent prognostic marker for NSCLC, yet we recommend that research on neovascularisation in NSCLC should be investigated for every histological subtype.

Recently, many studies suggesting an association between intratumoral vascularisation determined by vascularity stained for vWf staining and patient prognosis in patients with various malignant tumours have been published and the results obtained indicate that it may be an important prognostic marker [1–7]. However, there are many conflicting studies that suggest the opposite [12–14,24].

Why do opinions vary on this point? In the studies which suggested intratumoral vascularisation was a prognostic factor, vWf was used as a marker for staining microvessels and vWf staining to quantify microvasculature may be imprecise for several reasons [25]: (1) vWf is not expressed in all endothelial cells. The endothelial cells of microvessels are less rich in Weibel-Palade bodies than the endothelial cells of macrovessels; and these Weibel-Palade bodies can be stimulated by cytokines, such as thrombin and the interleukins, to release their stores of vWf. Thus, the endothelial cells of neocapillaries may be activated, releasing their vWf stores. (2) vWf is also present in lymphatic endothelium and in platelets. (3) In addition, it is impossible to distinguish newly formed vessels from older ones. For the above reasons, we believed that the accuracy of vWf staining as a method for determining the extent of tumoral microvascularisation is suspect, and thus, we investigated the efficacy of CD34.

CD34 antigen is expressed on immature human haematopoietic precursor cells and is progressively lost during maturation [26]. Although its function has not been elucidated, it is thought that CD34 might be involved in leucocyte adhesion or endothelial cell migration during angiogenesis [27]. In normal resting tissues, anti-CD34 antibodies predominantly stain the luminal endothelial membrane, whereas the abluminal membrane is negative or only weakly positive. In contrast, significant staining of the endothelial abluminal microprocesses (EAM) has been found in tumour stroma. In morphological studies on wound healing, tumour angiogenesis and embryogenesis, endothelial cells during the initial migration step of angiogenesis project abluminal extensions into the surrounding interstitium [28]. It has been shown that CD34 is a marker for EAM present during angiogenesis and that the antigenicity of CD34 is preserved by treating tissues with various fixatives such as freezing, ethanol, formalin or B5 [29]. In addition, it has been reported that during the carcinogenesis of bronchogenic carcinoma, microvessels stained for CD34 progressively increase from normal to hyperplastic, metaplastic and dysplastic bronchial epithelium and *in situ* carcinoma cells [30]. These facts suggest that CD34 is a suitable target for staining in the quantification of tumour vascularisation in lung cancer.

To the best of our knowledge, our study is the first report to evaluate microvascularisation using CD34 as an endothelial marker in patients with NSCLC. Our study demonstrated that the MVD determined by CD34 staining was higher in the VEGF positive group than in the VEGF negative group, but produced exactly the opposite results for vWf staining. Moreover, in comparing vWf to CD34, we found significant differences in the intensity of microvessel staining between them, misleading low vWf for low MVD.

Few studies have compared MVD determined by vWf staining with that determined by CD34 staining [15,16,31–33] . In these reports, the MVDs significantly correlated between the two markers in non-malignant tissues, but there was no correlation in malignant tissues. These findings also suggest that CD34 is more sensitive and specific than vWf for staining endothelial cells induced by tumour neovascularisation. In fact, MVD determined by CD34 staining related to survival in adenocarcinoma, distant metastasis and postoperative recurrence, whereas that determined by vWf staining did not.

No association was found between MVD and lymph node metastasis in our study; this is in conflict with other reports [7–10,34–38]. Recently, it has been proposed that VEGF increases the opportunity for nodal metastasis through neoblood and neolymphatic vessels [39]. Many of the prior studies that found a prognostic value of MVD determined by vWf staining may have done so as a result of the difficulty in distinguishing between blood and lymphatic microvessels in advanced cancer. Therefore, lymphatic vessels might be confused with blood vessels and counted as neoblood microvessels. Furthermore, in interpreting these studies, it is important to recognise that each group of investigators had to establish their own set of internal standards for quantifying the microvessels, had to determine their own cut-off value, and had to select a method to quantify the expression of VEGF.

With respect to early stage NSCLC, which is not influenced by infection, oppression and/or necrosis, postoperative distant metastasis appeared in all patients that were both VEGF positive and had a high MVD determined by CD34 staining. We suggest that the microvessels that responded to VEGF expression and were responsible for metastasis were stained not by vWf but by CD34. However, if vWf stained both blood and lymphatic vessels, the microvasculature stained by vWf must be a more suitable overall prognostic marker if we include both haematogenous and lymphogenous metastases. In fact, many of the previous studies established

the utility of vWf as a prognostic marker in predicting metastasis, but this may just be the result of vWf recognising not only neoblood vessels but also neolymphatic vessels.

We conclude that CD34 may be more indicative of tumoral microvasculature and more sensitive for newly formed microvessels than vWf in NSCLC. Recently, it has been shown that low VEGF expression and/or low MVD are linked to resistance to anticancer drugs [40]. Given this, the results of our study suggest that those patients with resectable stage I or stage II NSCLC who have both high MVD and high expression of VEGF might be good candidates for adjuvant chemotherapy and may be candidates for any anti-angiogeneic drugs developed in the future.

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