

ORIGINAL ARTICLE

Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB–IIA non-small cell lung cancer

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Aims: To evaluate the prognostic impact of tumour angiogenesis assessed by vascular endothelial growth factor (VEGF), microvessel density (MVD), and tumour vessel invasion in patients who had undergone radical resection for stage IB–IIA non-small cell lung cancer (NSCLC).

Methods: Fifty one patients (42 men, nine women; mean age, 62.3 years; SD, 6.9) undergoing complete surgical resection (35 lobectomy, 16 pneumonectomy) of pathological stage IB (n = 43) and IIA (n = 8) NSCLC were evaluated retrospectively. No patient underwent postoperative chemotherapy or neoadjuvant treatment. Tumour specimens were stained for VEGF and specific MVD markers: CD31, CD34, and CD105.

Results: VEGF expression significantly correlated with high CD105 expression ($p < 0.0001$) and tumour vessel invasion ($p = 0.04$). Univariate analysis showed that those patients with VEGF overexpression ($p = 0.0029$), high MVD by CD34 ($p = 0.0081$), high MVD by CD105 ($p = 0.0261$), and tumour vessel invasion ($p = 0.0245$) have a shorter overall survival. Furthermore, multivariate Cox regression analysis showed that MVD by CD34 ($p = 0.007$), tumour vessel invasion ($p = 0.024$), and VEGF expression ($p = 0.042$) were significant predictive factors for overall survival. Finally, the presence of both risk factors, tumour vessel invasion and MVD by CD34, was highly predictive of poor outcome (odds ratio, 3.4; 95% confidence interval, 1.7 to 6.5; $p = 0.0002$).

Conclusions: High MVD by CD34 and tumour vessel invasion are more closely related to poor survival than the other neoangiogenic factors in stage IB–IIA NSCLC. This may be because these factors are more closely related to the metastatic process.

Tumour neoangiogenesis has recently been recognised to be important in defining subsets of patients with poor outcome in cancer.^{1–3} Several reports^{4–15} have stated that the presence of neoangiogenesis is a significant factor in terms of overall and disease free survival in lung cancer. Immunohistochemical staining has been used to analyse vasculature markers such as vascular endothelial growth factor (VEGF)⁵ and microvessel density (MVD), as determined by CD31,⁶ CD34,⁷ and CD105 expression, in archival tumour material.¹⁶ VEGF is a glycoprotein with potent angiogenic, mitogenic, and vascular permeability enhancing activity in endothelial cells. CD31, CD34, and CD105 are endothelial antigens that have been used to highlight the density of intratumorous vessels as a direct marker of the degree of neoangiogenesis. Recently, CD105 proved to be superior to CD34 and CD31 in the evaluation of angiogenesis in non-small cell lung cancer (NSCLC)¹⁵ because it has a greater affinity for activated endothelial cells, whereas CD34 and CD31 can react with both normal vessels and activated vessels.

“Several reports have stated that the presence of neoangiogenesis is a significant factor in terms of overall and disease free survival in lung cancer”

Many patients with early TNM stage have a high probability of recurrence. Despite radical surgery, survival rate ranges between 40% and 70%.^{17–18} Failure is mainly the result of distant recurrences. To date, conventional parameters including analysis of performance status, histology subtype, size of the primary tumour, differentiation grading,

and mitotic rate have been investigated with different results.^{19–20}

Drawing from this background, we hypothesise that the rate of neoangiogenesis may be helpful in discriminating the probability of a poor prognosis in early stage NSCLC after radical surgery.

PATIENTS AND METHODS

Inclusion criteria

Subjects selected for our study were patients who had radical surgery for NSCLC at pathological stage IB (T2N0M0) or IIA (T1N1M0). We combined stages IB and IIA because of their similar prognosis.^{17–18} The patients were staged according to operative and pathological findings based on the AJCC/UICC TNM classification and stage grouping.¹⁷ N-factor was assessed on lymph nodes removed during routine mediastinal lymphadenectomy.

A preoperative staging computed tomography scan was performed in all patients. Only enlarged (greater than 1.5 cm in the maximal diameter)²¹ mediastinal lymph nodes were sampled preoperatively, either by mediastinoscopy or video thoracoscopy. The histology of each specimen was assessed according to the World Health Organisation classification,²² and the pathological stage of each tumour was recorded using the TNM staging system. Histology grading and N-stage were performed on haematoxylin and eosin stained sections.

Abbreviations: CI, confidence interval; MVD, microvessel density; NSCLC, non-small cell lung cancer; OR, odds ratio; TBS, trisphosphate buffered saline; VEGF, vascular endothelial growth factor

Patients who did not survive beyond 60 days after surgery were not included in our study to avoid bias from perioperative death. Patients who underwent minimal resection were ruled out from our present analysis.

In addition, only patients who had not received chemotherapy or radiotherapy before and after surgical resection were studied.

Study population

A retrospective study was undertaken in 51 consecutive patients (42 men and nine women), with a mean age of 62.3 years (SD, 6.9; range, 35–75) and stage IB (T2N0) or IIA (T1N1) NSCLC who underwent radical surgical resection at the thoracic surgery department of the Tor Vergata University, Italy between August 1990 and September 1998. The study project was submitted and approved by the human tissue use committee of the university. Tumour histology showed 28 squamous cell carcinomas, 19 adenocarcinomas, and four large cell carcinomas. Table 1 summarises the data.

In 16 patients the tumour was resected by pneumonectomy and in 35 by lobectomy.

Methods

Pathological investigations were carried out at the division of pathological anatomy and histology of the Tor Vergata University of Rome and of the University Campus Biomedico of Rome, Italy. NSCLC tumour specimens were fixed in 10% neutral buffered formaldehyde and embedded in paraffin wax. We selected one representative paraffin wax block from each case. Consecutive 4 µm thick sections were re-cut from each study block and used for the immunohistochemical study.

Immunohistochemical staining for VEGF, CD31, CD34, and CD105 was performed by the streptavidin–biotin method. In brief, sections were dewaxed and microwave treated at 500 W for five minutes twice in 10mM sodium citrate (pH 6.0). Endogenous peroxidase was blocked by incubation in 0.03% hydrogen peroxide in absolute methanol for 30 minutes at room temperature.

The antibodies used were: a polyclonal antibody against VEGF protein (A-20; 1/200 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA) at room temperature for two hours; a mouse monoclonal antibody against CD31 (JC70; 1/50 dilution; NeoMarkers, Fremont, California, USA) at room temperature for 30 minutes; a mouse monoclonal antibody against CD34 protein (QBEN 10; 1/50 dilution; Dako A/S, Glostrup, Denmark) at room temperature for 30 minutes; and mouse monoclonal antibody against

CD105 protein (105CO2; 1/50 dilution; NeoMarkers) at room temperature for 30 minutes. The optimal working dilutions were defined on the basis of a titration experiment. Negative controls for each tissue section were prepared by omitting the primary antibody.

After washing three times with trisphosphate buffered saline (TBS), sections were incubated with biotinylated goat antimouse or antirabbit immunoglobulin G (Dako A/S) for 10 minutes. They were then washed three times with TBS, treated with streptavidin–peroxidase reagent (Dako A/S) for 10 minutes, and then washed with TBS three times again. Finally, specimens were incubated in diaminobenzidine for five minutes, followed by haematoxylin counterstaining. Two experienced investigators (CR and AB) examined the slides, without knowledge of the corresponding clinicopathological data.

The expression of VEGF was assessed according to the percentage of immunoreactive cells in a total of 1000 neoplastic cells (quantitative analysis). The cutoff point to distinguish low from high VEGF expression was 25% of positive carcinoma cells.^{5 21 22} There was > 95% agreement between the two observers for the VEGF evaluation. A final score was determined by consensus after re-examination.

MVD was assessed using the criteria of Weidner *et al.*²³ The areas of highest neovascularisation were identified as regions of invasive carcinoma with the highest numbers of discrete microvessels stained for CD31, CD34, and CD105. Any brown stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumour cells, and other connective tissue elements was considered a single, countable microvessel. Microvessels in sclerotic areas within the tumour, where microvessels were sparse, and immediately adjacent areas of unaffected lung tissue were not considered in vessel counts. Each count was expressed as the highest number of microvessels identified within 0.3 mm² fields at a magnification of ×400. At least two fields were analysed for each tumour. All counts were performed by two investigators simultaneously, using a doubleheaded light microscope; both had to agree on what constituted a single microvessel before a vessel was included in the count.

Finally, tumour vessel invasion was assessed by identifying neoplastic emboli within the tumour vessels stained by CD34.⁵

Follow up

Follow up continued until death or at least until three years from the date of treatment. The survival time was calculated from the date of surgery to the date of death; relapse time was calculated from the date of surgery to the date of detection of local recurrence or systemic metastases.

Statistical analysis

All data were analysed using the SPSS software program (SPSS® 9.05 for Windows 1998; SPSS Inc, Chicago, Illinois, USA). The χ^2 test and Fisher's two tailed exact test were applied to assess the correlation between immunoreactivity and clinicopathological factors. Correlations between the different variables were adjusted for multiple comparisons. Survival was calculated from the time of surgery to the last date of follow up by means of the Kaplan-Meier estimate and prognosis was compared using the generalised Wilcoxon's analysis. Continuous variables of neoangiogenesis were split into categorical variables for the presentation of the results, but we performed the statistical analysis of survival before the recategorisation. The cutoff point to distinguish low from high VEGF expression was 25% of positive carcinoma cells,^{5 24 25} whereas for MVD the median value found in the study group was used.

Table 1 Study population features divided according to stage

Conventional risk factors	Total	%
Sex		
Male	42	82.3%
Female	9	17.7%
Smoker		
No	6	11.7%
Yes	45	88.3%
Surgical procedure		
Lobectomy	35	68.6%
Pneumonectomy	16	31.4%
Histology		
Squamous	28	54.9%
Non-squamous	23	45.1%
Grading		
G1	11	21.5%
G2	22	43.1%
G3	18	35.4%

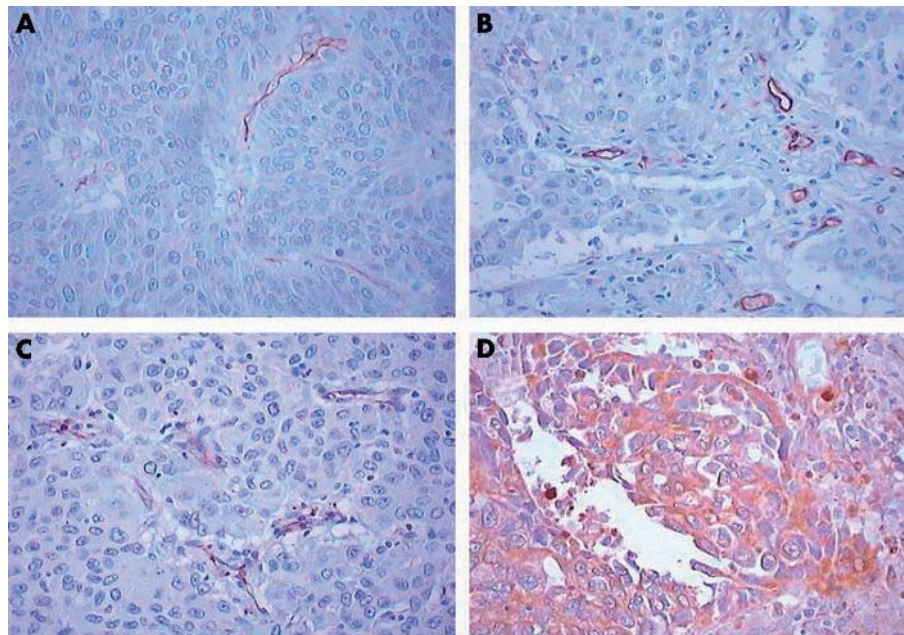


Figure 1 Representative immunostaining for (A) vascular endothelial growth factor, (B) CD31, (C) CD34, and (D) CD105.

Table 2 Immunohistochemical parameters in patients with NSCLC

	Number	%
CD34		
Low	21/51	41.2%
High	30/51	58.8%
CD105		
Low	17/51	33.3%
High	34/51	66.7%
CD31		
Low	20/51	39.2%
High	31/51	60.8%
VEGF		
Low	9/51	17.6%
High	42/51	82.4%
Tumour vessel invasion		
No	38/51	74.5%
Yes	13/51	25.5%

NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor.

Factors that were significant on univariate analysis were entered into multivariate analysis using the Cox stepwise logistic regression test to investigate the independence of the various risk factors.

RESULTS

Median follow up of selected patients was 48.1 months (range, 4–150). Median survival was 54 months. At the end of our study, 29 patients had died, 23 of them directly from NSCLC after locoregional (n = 5) or distant (n = 18) relapse. The overall three year survival rate was 58.8% and five year survival was 51.1%, lower than that reported by Mountain¹⁷ (57% and 55%) for pathological stages IB and IIA, respectively.

Table 2 shows the immunohistochemical expression of the angiogenetic factors evaluated. The median VEGF rate was 75.1% (range, 2–98%). High expression was seen in 42 patients. The median rate of MVD as assessed by CD34 was 126.8 (range, 62–225), and the value of 130 was chosen as

Table 3 Correlation between angiogenetic factors and conventional risk factors

	VEGF		CD34		CD31		CD105		Vessel invasion	
Stage										
IB	8	35	20	23	17	26	16	27	30	13
IIA	1	7	1	7	3	5	1	7	8	0
χ^2 Value (p value)	0.008 (0.929)		1.970 (0.160)		0.082 (0.775)		0.908 (0.341)		1.849 (0.174)	
Smoker										
No	1	5	3	3	2	4	2	4	6	1
Yes	8	37	18	27	18	27	15	30	32	12
χ^2 Value (p value)	0.253 (0.615)		0.001 (0.979)		0.017 (0.896)		0.213 (0.645)		0.070 (0.791)	
Surgical procedure										
Lobectomy	7	28	12	23	11	24	10	25	27	8
Pneumonectomy	2	14	9	7	9	7	7	9	11	5
χ^2 Value (p value)	0.066 (0.798)		1.374 (0.241)		1.892 (0.169)		0.558 (0.455)		0.085 (0.770)	
Histology										
Squamous	4	24	9	19	13	15	10	18	21	7
Non-squamous	5	18	12	11	7	16	7	16	17	6
χ^2 Value (p value)	0.106 (0.745)		1.347 (0.246)		0.767 (0.381)		0.010 (0.921)		0.055 (0.815)	
Grading										
G1	4	7	9	2	5	6	5	6	10	1
G2	3	19	8	14	8	14	5	17	17	5
G3	2	16	4	14	7	11	7	11	11	7
χ^2 Value (p value)	3.424 (0.180)		1.382 (0.876)		0.255 (0.880)		2.091 (0.352)		3.347 (0.188)	

VEGF, vascular endothelial growth factor.

Table 4 Correlation between vascular endothelial growth factor (VEGF) expression and the other neoangiogenetic factors

	VEGF		χ^2	p Value
	Low	High		
CD34				
Low	6 (12%)	15 (29%)	1.793	0.181 (NS)
High	3 (6%)	27 (53%)		
CD31				
Low	5 (10%)	15 (29%)	0.533	0.465 (NS)
High	4 (8%)	27 (53%)		
CD105				
Low	8 (16%)	9 (18%)	12.295	<0.0001
High	1 (2%)	33 (64%)		
Vessel invasion				
No	9 (18%)	29 (56%)	2.297	0.04
Yes	0 (0%)	13 (26%)		

Table 5 Survival and immunohistochemical parameters in patients with NSCLC analysed by univariate analysis

	Median survival (months)	95% CI	p Value
CD 34			
Low	92	65 to 120	0.0081
High	24	8 to 40	
CD105			
Low	100	68 to 131	0.0261
High	34	9 to 59	
CD31			
Low	90	40 to 140	0.0429
High	26	8 to 44	
VEGF			
Low	128	101 to 154	0.0029
High	34	18 to 50	
Tumour vessel invasion			
No	85	51 to 119	0.0245
Yes	24	19 to 29	

CI, confidence interval; NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor.

the cutoff point. High expression was seen in 30 patients. The median rate of MVD as assessed by CD31 was 84.6 (range, 57–108) and the value of 80 was chosen as the cutoff point. The median rate of MVD as assessed by CD105 was 57.6 (range, 42–89), and the value of 60 was chosen as the cutoff point. Tumour vessel invasion was present in 13 patients. Figure 1 shows representative examples of staining for CD31, CD34, CD105, and VEGF.

None of the conventional factors taken into consideration (age more than 60, sex, type of resection, smoking habit, non-squamous histology, undifferentiated grading, presence of N1 lymph nodes) was significantly related to

longterm survival or to the angiogenetic factors examined (table 3).

Analysis of the correlation between VEGF expression and the other neoangiogenetic factors showed that VEGF overexpression was significantly correlated with high CD105 ($p < 0.0001$) and tumour vessel invasion ($p = 0.04$) (table 4).

Univariate analysis, performed by five year Kaplan-Meier survival rate for dichotomised variables, showed a significant association with VEGF overexpression ($p = 0.0029$), with

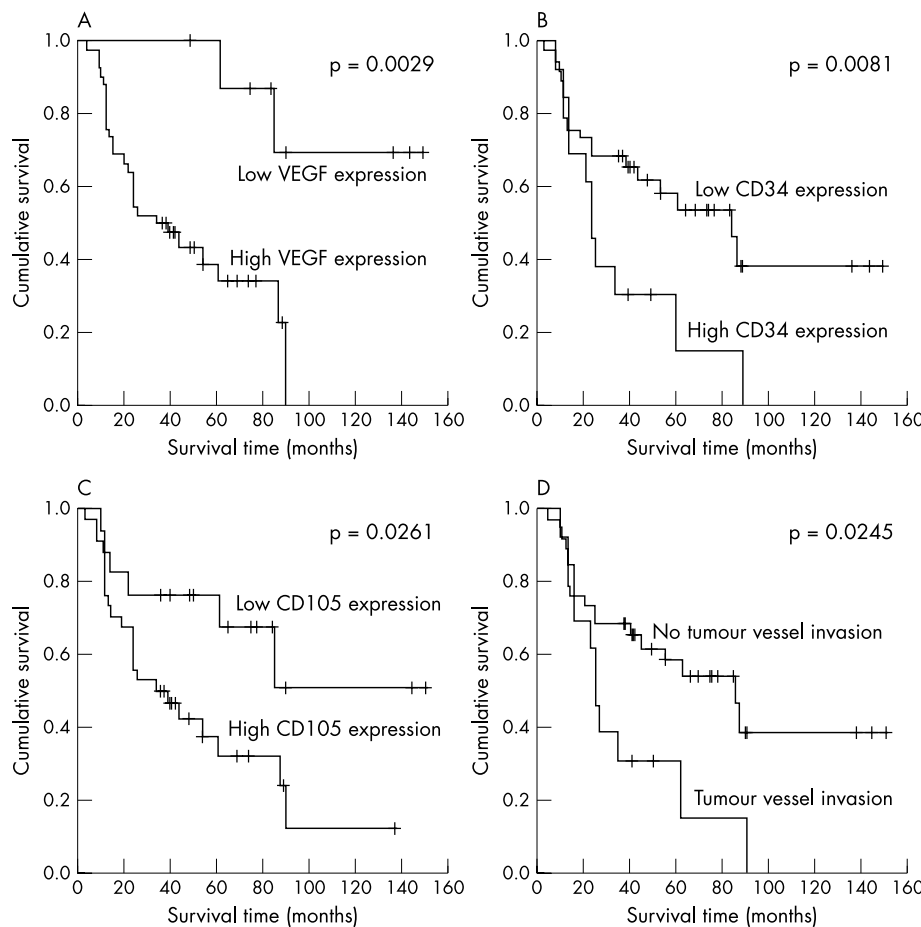


Figure 2 Kaplan-Meier curve for the study population stratified for the various neovascularisation factors. (A) VEGF expression. The five year overall survival rate was 88% for low expression ($\leq 25\%$) and 39.2% for overexpression ($> 25\%$). (B) Microvessel density (MVD) assessed by CD34. The five year overall survival rate was 69.6% for low (≤ 130) and 29.5% for high (> 130) MVD. (C) MVD assessed by CD105. The five year overall survival rate was 76.5% for low (≤ 60) and 37.7% for high (> 60) MVD. (D) Tumour vessel invasion. The five year overall survival rate was 30.9% when present and 60.9% when absent.

Table 6 Multivariate Cox regression analysis of overall survival in patients with NSCLC

	RR of death	95% CI	p Value
CD34			
Low	1	–	
High	3.501	1.408 to 8.702	0.007
CD105			
Low	1	–	
High	1.141	0.429 to 3.036	0.792
CD31			
Low	1	–	
High	2.225	0.950 to 5.214	0.066
VEGF			
Low	1	–	
High	3.617	1.054 to 14.61	0.042
Tumour vessel invasion			
No	1	–	
Yes	2.817	1.148 to 6.913	0.024

CI, confidence interval; NSCLC, non-small cell lung cancer; RR, relative risk; VEGF, vascular endothelial growth factor.

high MVD by CD34 (p = 0.0081) and by CD105 (p = 0.0261), and with tumour vessel invasion (p = 0.0245). These data are summarised in table 5 and fig 2.

Multivariate Cox regression analysis showed that MVD as assessed by CD34 (odds ratio (OR), 3.5; 95% confidence interval (CI), 1.408 to 8.702; p = 0.007), tumour vessel invasion (OR, 2.8; 95% CI, 1.148 to 6.913; p = 0.024), and VEGF expression (OR, 3.6; 95% CI, 1.054 to 14.61; p = 0.042) were significant predictive factors for overall survival (table 6).

The presence of both risk factors, tumour vessel invasion and MVD as assessed by CD34, was highly predictive of poor outcome (p = 0.0002; OR, 3.4; 95% CI, 1.7 to 6.5). These data are shown in table 7 and fig 3.

DISCUSSION

Angiogenesis is an essential process in the progression of malignant tumours. A variety of proteins, including growth factors and extracellular matrix enzymes, have been recognised to be potent inducers of angiogenesis.²⁶ Recent evidence suggests that tumour angiogenesis is associated with patient outcome in several malignancies. Therefore, neoangiogenesis may become an integral part of a more consistent staging system. The first study correlating MVD with prognosis in NSCLC was that of Macchiarini and colleagues⁴ in 1992, who assessed neovascularisation by the use of anti-factor VIII. Later, factor VIII,^{6 27} CD34,^{9 11} and VEGF expression,^{5 10 12-14 28 29} in addition to non-vascular growth factors³⁰ were investigated. Comparisons and meta-analysis are very difficult to perform because of the different methodologies and evaluation criteria for VEGF and MVD used, in addition to the heterogeneity of the study samples (table 8).

Such different results highlight the need for more reliable markers of neoangiogenesis. Nevertheless, recently Meert

Table 7 Survival and combination of CD34 and tumour invasion in patients with NSCLC

Groups	Median survival (months)	95% CI	p Value
A (CD34+/TVI+)	15	7 to 23	<0.0001
B (CD34- /TVI+, CD34+/TVI-)	44	6 to 82	
C (CD34- /TVI-)	116	84 to 148	

CI, confidence interval; NSCLC, non-small cell lung cancer; TVI, tumour vessel invasion.

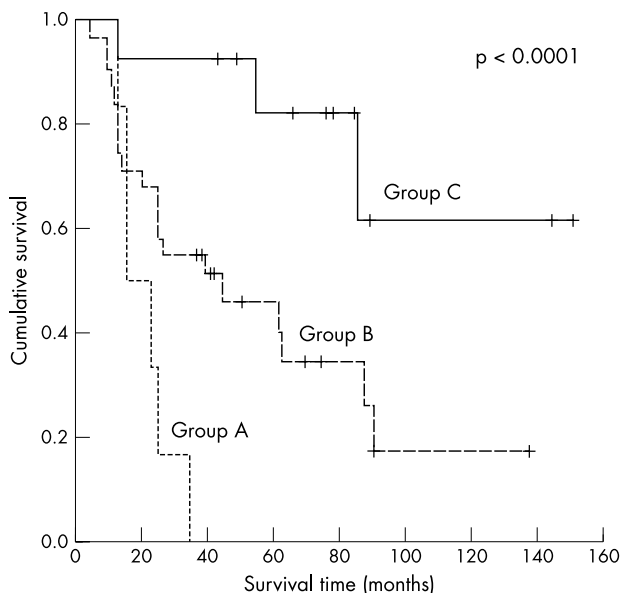


Figure 3 Kaplan-Meier curve for the study population for combined tumour vessel invasion and microvessel density as assessed by CD34. The five year overall survival rate was 77.3% for no positive factor, 43.2% for one positive factor, and 0% for two positive factors.

and colleagues²⁶ performed a meta-analysis based on a systematic review of the literature to assess the prognostic value on survival of microvessel count in patients with lung cancer. They found that a high MVD was a significantly poor prognostic factor for survival in NSCLC, however it was assessed (factor VIII, CD34, or CD31).

The purpose of our study was to identify the best markers of neoangiogenesis and correlate them to survival in a group of patients with early stage NSCLC. To the best of our knowledge, this is the first study dealing with such a wide panel of factors in such a selected group of patients.

Our data show that patients classified at the same prognostic stage according to classic morphological criteria have very variable prognoses. Within this group, we found that some markers of neoangiogenesis were useful tools to characterise patients with a poor outcome.

“Interestingly, CD105 proved to be a significant marker of neoangiogenesis, but it was excluded at multivariate analysis”

Our results show that high VEGF expression, high MVD as assessed by CD105 and CD34, and tumour vessel invasion are good markers of angiogenesis and are significantly correlated with a poor survival rate. Nevertheless, at multivariate analysis, only MVD assessed by CD34 and tumour vessel invasion were selected by the statistical model. The combination of these two markers classified three populations with different risks of dying.

Interestingly, CD105 proved to be a significant marker of neoangiogenesis, but it was excluded at multivariate analysis. This finding contrasts with the results of Tanaka *et al*,¹⁵ who found that CD105 expression was the best marker of angiogenesis and was a significant prognosticator of disease free survival, superior to CD34. This apparent contradiction might be explained by the fact that CD34 and tumour vessel invasion are more strictly related to the metastatic process than neoangiogenesis by itself. Yano *et al* have recently demonstrated a higher incidence of distant metastases and a shorter survival in patients with high grade MVD as assessed

Table 8 Studies correlating neoangiogenesis and survival in NSCLC

First author	Year	Number of patients	Stage	Factor	Correlation
Giatromanolaki ⁶	1997	134	I, II	CD31	NS
Pastorino ⁸	1997	137	I	CD31	NS
Fontanini ⁹	1997	407	I-III	CD34	S
Duarte ²⁶	1998	106	I	VIII, CD31	S, NS
Imoto ²⁷	1998	91	I-III	VEGF	S
Matsuyama ¹¹	1998	101	I-III	CD34	S
Decaussin ¹²	1999	81	I, II	VEGF	NS
Kakolyris ¹³	1999	69	I, II	CD31	S
Yano ⁵	2000	108	I-IV	CD34, VEGF	S, NS
Koukourakis ¹⁰	2001	102	I, II	VEGF	S
Han ¹⁴	2001	85	I	VEGF	S
Offersen ⁷	2001	143	I-III	VEGF, CD34	NS, NS
Tanaka ¹⁵	2001	236	I-III	CD105, CD34	S, NS

NS, not significant; NSCLC, non-small cell lung cancer; S, significant; VEGF, vascular endothelial growth factor.

Take home messages

- Patients with non-small cell lung cancer with vascular endothelial growth factor (VEGF) overexpression, high microvessel density (MVD) by CD34 and CD105, and tumour vessel invasion had shorter overall survival on univariate analysis
- Multivariate analysis showed that MVD by CD34, tumour vessel invasion, and VEGF expression were significant predictive factors for overall survival
- The presence of both tumour vessel invasion and MVD by CD34 was highly predictive of poor outcome and might be useful to identify a subset of high risk patients who could be targeted for more aggressive treatment

by CD34.⁵ A significant correlation was also shown between CD34 and tumour vessel invasion.

The limitations of our study are the small sample size and the length of the minimum follow up required. As far as the size of the study group is concerned, we chose only a particular subset of patients (stages IB–IIA) to avoid bias resulting from different prognoses. Indeed, even if these patients are staged into different classes the prognosis is similar.^{17, 18} Conversely, stage I, compounded with IA and IB, is a heterogeneous group. Nevertheless, despite the relatively short follow up period, we were able to demonstrate a significant impact of the expression of the analysed factors on survival.

CONCLUSIONS

We have confirmed previous work claiming that VEGF, CD105, and CD34 are excellent markers of neoangiogenesis in NSCLC (table 4). In our study, the overexpression of these factors correlated with poor prognosis in a homogeneous group of patients who underwent radical surgery. The two factors indicative of the metastatic process, CD34 and tumour vessel invasion, correlated closely with poor prognosis, and their combination may be useful to stratify patients into three different populations with a low, intermediate, and high risk of short survival.³⁰

Our research group is presently investigating the expression of several angiogenic markers on a wider sample of NSCLCs to provide a better definition of their potential prognostic value. The identification of a high risk subset of patients may be useful so that these patients can be given adjuvant therapy (although the role of such treatment is presently still debatable) or a targeted and specific treatment in the near future.³¹

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