Inverse Prognostic Impact of Angiogenic Marker Expression in Tumor Cells versus Stromal Cells in Non – Small Cell Lung Cancer

Tom Donnem,[1,3] Samer Al-Saad,[2,4] Khalid Al-Shibli,[2,5] Marit P. Delghandi,[1,3] Magnus Persson,[2]
Marit N. Nilsen,[2] Lill-Tove Busund,[2,4] and Roy M. Bremnes[1,3]

Abstract **Purpose:** The vascular endothelial growth factors (VEGF-A, -C, -D) and the VEGF receptors (VEGFR-1, -2, and -3) are important molecular markers in angiogenesis and lymphangiogenesis. This study elucidates the prognostic significance of these molecular markers in tumor cells as well as in the tumor stroma of resected non – small cell lung cancer tumors.
Experimental Design: Tumor tissue samples from 335 resected patients with stage I to IIIA disease were obtained and tissue microarrays were constructed from duplicate cores of tumor cells and surrounding stromal tissue from each resected specimen. Immunohistochemistry was used to evaluate the expression of each molecular marker. Microvessel density was assessed by CD34 immunohistochemical staining.
Results: In univariate analyses, high tumor cell expression of VEGF-A ($P = 0.0005$), VEGFR-1 ($P = 0.013$), VEGFR-2 ($P = 0.006$), and VEGFR-3 ($P = 0.0003$) were negative prognostic indicators for disease-specific survival (DSS). In tumor stroma, however, high expression of VEGF-A ($P = 0.017$), VEGF-C ($P = 0.003$), VEGF-D ($P = 0.009$), VEGFR-1 ($P = 0.01$), and VEGFR-2 ($P = 0.019$) correlated with good prognosis. There was no significant correlation between microvessel density and DSS. In multivariate analyses, high expression in tumor cells of VEGFR-3 ($P = 0.007$) was an independent negative prognostic factor for DSS, whereas in stromal cells, high VEGF-C ($P = 0.004$) expression had an independent positive survival impact.
Conclusion: These are the first tissue microarray data in non – small cell lung cancers showing a positive prognostic impact by highly expressed angiogenic markers in tumor stroma, with VEGF-C as a major independent prognostic indicator.

The non – small cell lung cancers (NSCLC) comprise 80% of lung cancers and the vast majority of these patients present with advanced disease and have a grim prognosis (1, 2). Of the 25% which are considered candidates for curative surgery at diagnosis (stage I-IIIA), 65% will relapse within 2 years and subsequently die of metastatic spread (3, 4).

To improve the clinical outcome of patients with NSCLC, elucidating the mechanisms of tumor biology is considered most important. As the malignant tumor requires angiogenesis to grow beyond 1 to 2 mm^3 in size or to metastasize (5), angiogenesis is regarded to be one of the hallmarks of cancer development (6). Thus, the inhibition of tumor angiogenesis is a pivotal therapeutic strategy. Already, some antiangiogenic agents are available for clinical therapy and more are in the pipeline (7).

Authors' Affiliations: Institute of [1]Clinical Medicine and [2]Medical Biology, University of Tromsö, Departments of [3]Oncology and [4]Pathology, University Hospital of Northern Norway, Tromsö, Norway, and [5]Department of Pathology, Nordland Central Hospital, Bodö, Norway
Received 2/19/07; revised 8/6/07; accepted 8/14/07.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Tom Donnem, Department of Oncology, Institute of Clinical Medicine, University of Tromsö, 9037 Tromsö, Norway. Phone: 47-7762-6000/47-7764-5427; Fax: 47-7762-6779; E-mail: tom.donnem@fagmed.uit.no.
© 2007 American Association for Cancer Research.
doi:10.1158/1078-0432.CCR-07-0414

Various angiogenic mechanisms may be differentially important in different tumor types and/or stages of neoplastic progression (8). However, one of the major pathways involved in angiogenesis is the vascular endothelial growth factor (VEGF and VEGFR) family of proteins and receptors (9). This family comprises six secreted glycoproteins of which VEGF-A, VEGF-C, and VEGF-D are of great significance (8, 10). These VEGF ligands mediate their angiogenic effect via the receptor tyrosine kinases VEGFR-1 (flt-1), VEGFR-2 (KDR or Flk-1), and VEGFR-3 (Flt-4; refs. 11 – 13). VEGF-A has been regarded as the major player for angiogenesis. It binds to VEGFR-1 and VEGFR-2, of which VEGFR-2 is the major mediator of the mitogenic and angiogenic effects of VEGF-A. VEGF-C and VEGF-D activate VEGFR-3 and seem to be important for lymphatic endothelial cell growth, migration, and survival. However, proteolytically processed VEGF-C and VEGF-D can also induce blood vessel growth by activating VEGFR-2 (14, 15). VEGFR-3 deletion leads to defects in blood vessel remodeling and embryonic death at midgestation, indicating that activation of VEGFR-3 might promote both angiogenesis and lymphangiogenesis (16, 17).

These angiogenic markers are important in the interaction between a diversity of stromal and tumor cells (18). In tumor development, the angiogenic switch is associated with the onset of expression and secretion of angiogenic factors by tumor cells. The tumor cell secretion of growth factors leads to a complex interplay with subsequent stromal secretion of growth factors and activation of endothelial cell receptors (19). Unlike normal

tissue, the tumor stroma contains increased amounts of inflammatory infiltrates, an increased microvessel density (MVD) with dysfunctional lymphatics and blood vessels, and a denser extracellular matrix with reactive fibroblasts (20). There is growing recognition that the tumor stroma plays a crucial role in tumorigenesis (21), but many questions regarding the cross-talk between stromal and tumor cells, as a part of the angiogenic process, remains to be answered.

To assess the prognostic significance of VEGF-A, -C and -D and their receptors VEGFR-1, -2, and -3, we examined the ligand and receptor expressions in both tumor cells and stroma of resected NSCLC tumors.

Patients and Methods

Patients and clinical samples. Primary tumor tissues from anonymized patients diagnosed with pathologic stage I to IIIA NSCLC at the University Hospital of Northern Norway and Nordland Central Hospital from 1990 to 2004 were used in this retrospective study. In total, 371 patients were registered from the hospital database. Of these, 36 patients were excluded from the study due to (*a*) radiotherapy or chemotherapy prior to surgery (n = 10), (*b*) other malignancy within 5 years prior to NSCLC diagnosis (n = 13), and (*c*) inadequate paraffin-embedded fixed tissue blocks (n = 13). Thus, 335 patients with complete medical records and adequate paraffin-embedded tissue blocks were eligible.

This report includes follow-up data as of September 30, 2005. The median follow-up was 96 (range, 10-179) months. Complete demographic and clinical data were collected retrospectively. Formalin-fixed and paraffin-embedded tumor specimens were obtained from the archives of the Departments of Pathology at University Hospital of Northern Norway and Nordland Central Hospital. The tumors were staged according to the International Union Against Cancer's tumor-node-metastasis classification and histologically subtyped and graded according to WHO guidelines (22). The National Data Inspection Board and The Regional Committee for Research Ethics approved the study.

Microarray construction. All lung cancer cases were histologically reviewed by two pathologists (S. Al-Saad and K. Al-Shibli) and the most representative areas of tumor cells (neoplastic cells) and tumor stroma were carefully selected and marked on the H&E slide and sampled for the tissue microarray blocks (TMA). The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments). The detailed methodology has been previously reported (23). Briefly, we used a 0.6-mm diameter stylet, and the study specimens were routinely sampled with two replicate core samples (different areas) of neoplastic tissue and two of tumor stroma. Normal lung tissue localized distant from the primary tumor were used as negative controls.

To include all core samples, eight tissue array blocks were constructed. Multiple 5-μm sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemistry analysis.

Immunohistochemistry. The applied antibodies were subjected to in-house validation by the manufacturer for immunohistochemical analysis on paraffin-embedded material. The antibodies used in the study were as follows: VEGF-A (1:10, rabbit polyclonal; RB-1678; Neomarkers), VEGF-C (1:25, rabbit polyclonal; 18-2255; Zymed Laboratories), VEGF-D (1:40, mouse monoclonal; MAB286; R&D Systems), VEGFR-1 (1:10, rabbit polyclonal; RB-1527; Neomarkers), VEGFR-2 (1:25, rabbit polyclonal; RB-9239; Neomarkers), and VEGFR-3 (1:10, rabbit polyclonal; Sc-321; Santa Cruz Biotechnology).

Sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was done by placing the specimen in 0.01 mol/L of citrate buffer at pH 6.0 and exposed to repeated (twice) microwave heating of 10 min (except VEGFR-3, twice for 5 min) at 450 W. VEGF-D was heated for 45 min in a water bath in 0.01 mol/L of citrate buffer. The DAKO EnVision+ System-HRP kit (diaminobenzidine) was used for endogen peroxidase blocking. As negative staining controls, the primary antibodies were replaced with the primary antibody diluent. Primary antibodies were incubated for 30 min in room temperature (except VEGFR-3, 20 min, and VEGF-D, overnight at 4°C). The DAKO EnVision+ System-HRP kit (diaminobenzidine) was used to visualize the antigens. This was followed by the application of liquid diaminobenzidine and substrate-chromogen, yielding a brown reaction product at the site of the target antigen. Finally, all slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative staining controls, all TMA stainings were done in a single experiment.

Immunohistochemical scoring. By light microscopy, representative and viable tissue sections were scored semiquantitatively for cytoplasmic staining. The dominant staining intensity in both tumor cells and stromal cells was scored as 0, negative; 1, weak; 2, intermediate; and 3, strong (Fig. 1). The cell density of the stroma was scored as 1, low density; 2, intermediate density; 3, high density (Fig. 1). All samples were anonymized and independently scored by two pathologists (S. Al-Saad and K. Al-Shibli). In case of disagreement, the slides were reexamined and a consensus was reached by the observers. In most tumor cores, as well as in some stromal cores, there are a mixture of stromal cells and tumor cells. However, by morphologic criteria they have only scored the staining intensity of tumor cells in tumor cores and the intensity and density of stromal cells in stromal cores. When assessing one variable for a given core, the observers were blinded to the scores of the other variables and to outcome. To evaluate the interindividual variability with respect to immunohistochemical scoring, 100 consecutive tumor cell cores and tumor stroma cores stained for VEGF-C and VEGFR-3, evaluated by two pathologists (S. Al-Saad and K. Al-Shibli), were examined. The mean score for duplicate cores from each individual was calculated separately in tumor cells and stroma, and high expression in tumor cells was defined as score ≥2 (VEGF-C, VEGF-D, VEGFR-2), or >2 (VEGF-A, VEGFR-1, VEGFR-3). Stromal expression was calculated by summarizing density score (1-3) and intensity score (0-3) prior to categorizing into low and high expression. High expression in stroma was defined as score ≥4 (except VEGF-A >4).

MVD. We assessed MVD by CD34 immunohistochemical staining and any stained endothelial cell or endothelial cell cluster separated from other stromal elements was considered as single countable microvessels. The MVD was defined as the number of microvessels identified within one array core (0.6 mm diameter), tumor or stromal MVD was scored as 0, negative; 1, 1-10 vessels per core; 2, 11-20 vessels per core; 3, >20 vessels per core. In tumor cores, only microvessels surrounded by viable tumor cells were counted, whereas in stromal cores, only microvessels adjacent to other stromal cells were scored. As for the VEGFs and VEGFRs, a mean score for duplicate cores from each individual was calculated separately in tumor cells and stroma. High MVD in tumor cores was defined as a mean score = 2.5 or 3, whereas in stromal cores, high MVD was defined as a mean score = 3.

Statistical methods. All statistical analyses were done using the statistical package SPSS, version 14. The immunohistochemistry scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results. The χ^2 test and Fishers exact test were used to examine the association between molecular marker expression and various clinicopathologic variables. Univariate analysis was done by using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log-rank test. Disease-specific survival (DSS) was determined from the date of surgery to the time of lung cancer death. To assess the independent value of different pretreatment variables on survival in the presence of other variables, multivariate analysis was carried out using the Cox proportional hazards model. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively.

Results

Clinicopathologic variables. Demographic, clinical, and histopathologic variables are shown in Table 1. The median age was 67 (range, 28-85) years and the majority of the patients were male (76%). The NSCLC tumors comprised 191 squamous cell carcinomas, 95 adenocarcinomas, 31 large-cell carcinomas, and 18 bronchioalveolar carcinomas. Due to nodal metastasis or nonradical surgical margins, 59 (18%) patients received postoperative radiotherapy.

Interobserver variability. Interobserver scoring agreement was tested for one ligand (VEGF-C) and one receptor (VEGFR-3). For VEGF-C: tumor r = 0.95, P < 0.001; stroma intensity r = 0.93, P < 0.001; stroma density r = 0.93, P < 0.001. For VEGFR-3: tumor r = 0.98, P < 0.001; stroma intensity r = 0.96, P < 0.001; stroma density r = 0.97, P < 0.001.

Expression of angiogenic markers and their correlations. Angiogenic marker expression was observed in the cytoplasm of tumor cells. Based on morphologic criteria, pneumocytes in control cores from normal lung tissue distant from the primary tumor showed no significant immunostaining. In tumor stroma and in control cores, inflammatory cells (macrophages, lymphocytes, granulocytes, and plasma cells) frequently showed positive staining, whereas fibroblast-like cells occasionally presented positive staining. No correlation was observed between tumor cell or stromal angiogenic marker expression and performance status, tumor differentiation, vascular infiltration, or histology (except high stromal expression of VEGF-C in large-cell carcinomas; P = 0.04). Tumor cell VEGFR-1 (P = 0.03) and VEGFR-3 (P = 0.02) overexpression was more common in patients younger than 65 years. With the exception of tumor cell VEGF-A expression (high expression; T_1 versus T_2 and T_3, 27% versus 49% and 41%, respectively; P = 0.001), no association was seen between T stage and expression rates.

No correlation was observed between tumor core MVD and VEGF or VEGFR expression. For stromal core MVD, there was a significant correlation between MVD and stromal VEGF-C (r = 0.11, P = 0.034) and between MVD and stromal VEGF-D (r = 0.17, P = 0.002).

Univariate analysis. In addition to clinical variables (Table 1), tumor cell expression of VEGF-A (P = 0.0005), VEGFR-1 (P = 0.013), VEGFR-2 (P = 0.006), and VEGFR-3 (P = 0.0003) and stromal expression of VEGF-A (P = 0.017), VEGF-C (P = 0.003), VEGF-D (P = 0.009), VEGFR-1 (P = 0.01), and VEGFR-2 (P = 0.019) were prognostic indicators for DSS (Table 2; Figs. 2 and 3). There was no significant association between tumor core MVD (P = 0.96) or stromal core MVD (P = 0.19) and DSS.

Multivariate Cox proportional hazards analysis. In multivariate analyses (Table 3), including clinicopathologic variables and angiogenic markers, tumor cell expression of VEGFR-3 (P = 0.007), stromal cell expression of VEGF-C (P = 0.004), performance status (P = 0.005), histologic differentiation (P = 0.018), T-stage (P = 0.003), N-stage (P = 0.003), and vascular infiltration (P = 0.042) were independent prognostic

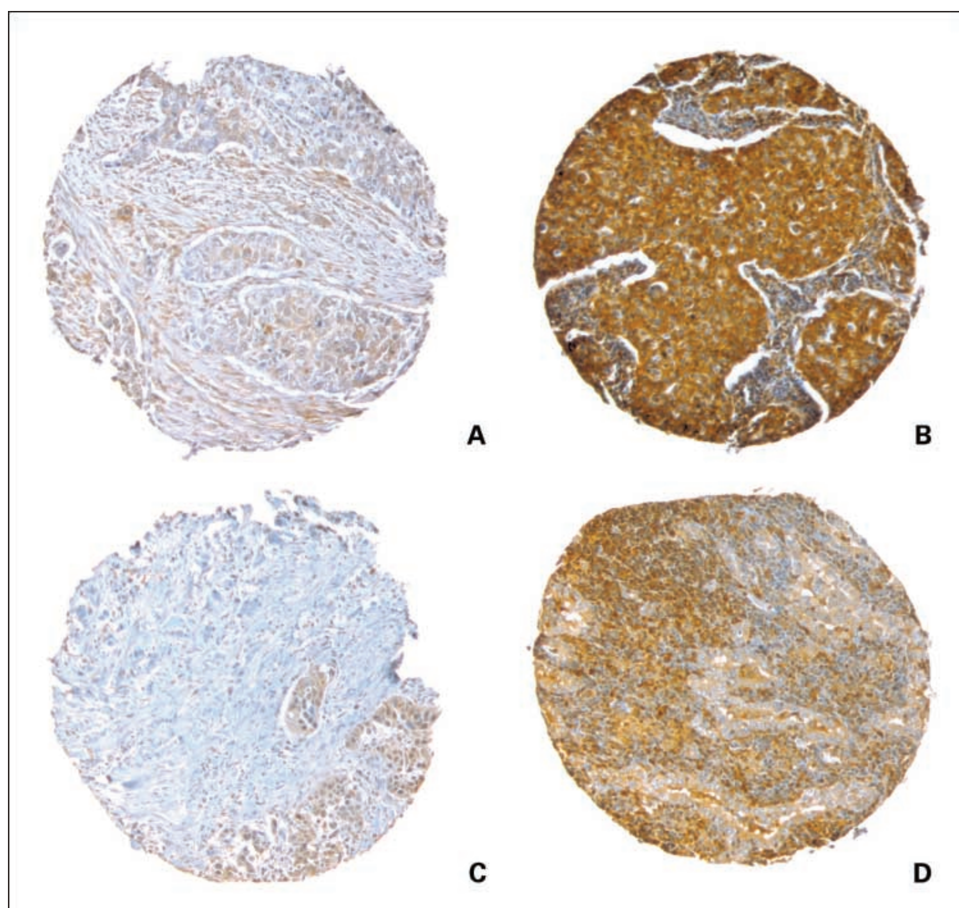

Fig. 1. Immunohistochemical analysis of TMA of NSCLC representing different scores for tumor cell VEGFR-3 and stromal VEGF-C. *A,* tumor cell VEGFR-3 score 1; *B,* tumor cell VEGFR-3 score 3; *C,* stromal VEGF-C low score (density 1, intensity 0); and *D,* stromal VEGF-C high score (density 3, intensity 3).

Table 1. Prognostic clinicopathologic variables as predictors for DSS in 335 patients with NSCLC (univariate analysis; log-rank test)

Characteristic	Patients (*n*)	Patients (%)	Median survival (mo)	5-y survival (%)	*P*
Age (y)					
≤65	156	47	104	57	0.62
>65	179	53	NR	58	
Sex					
Female	82	25	127	65	0.19
Male	253	75	84	55	
Smoking					
Never	15	5	19	43	0.13
Present	215	64	NR	60	
Previous	105	31	84	54	
Performance status					
Normal	197	59	NR	62	0.04
Slightly reduced	120	36	61	52	
In bed <50%	18	5	36	40	
Weight loss					
<10%	303	90	127	57	0.92
>10%	32	10	NR	57	
Histology					
SCC	191	57	NR	65	0.30
Adenocarcinoma	95	28	52	44	
BAC	18	5	NR	67	
LCC	31	9	84	54	
Differentiation					
Poor	138	41	48	48	0.001
Moderate	144	43	NR	64	
Well	53	16	NR	65	
Surgical procedure					
Lobectomy + wedge*	243	73	NR	61	0.0009
Pneumonectomy	92	27	35	46	
Stage					
I	212	63	NR	68	<0.0001
II	91	27	41	46	
IIIa	32	10	18	22	
Tumor status					
1	90	27	NR	75	0.002
2	218	65	84	52	
3	27	8	42	43	
Nodal status					
0	232	69	NR	66	<0.0001
1	76	23	37	43	
2	27	8	18	20	
Surgical margins					
Free	307	92	127	58	0.34
Not free	28	8	64	51	
Vascular infiltration					
No	284	85	NR	61	0.0005
Yes	51	15	25	35	
Postoperative radiotherapy					
No	276	82	NR	61	0.002
Yes	59	18	41	42	

Abbreviations: NR, not reached; SCC, squamous cell carcinoma; BAC, bronchioalveolar carcinoma; LCC, large-cell carcinoma.
*Wedge, *n* = 10.

factors. High expression of tumor cell VEGFR-2 tended towards an independent negative effect on survival, but did not reach statistical significance ($P = 0.085$).

Discussion

We present the first large-scale study using high-throughput TMA analyses to examine the prognostic impact of both tumor cell and stromal VEGF-A, -C, and -D and VEGFR-1, -2, and -3 in an unselected population of surgically resected NSCLC patients. Interestingly, stromal VEGF-A, -C, -D and VEGFR-1 and -2 all showed a significant positive correlation between expression and prognosis, quite contrary to the inverse association between tumor cell angiogenic marker expression and prognosis. In multivariate analysis, high stromal expression of VEGF-C correlated with a good prognosis, whereas high tumor cell expression of VEGFR-3 was a negative prognostic indicator for DSS.

TMA for immunophenotyping of malignant tumors has been validated (24) and used in lung cancer research (23).

Although the presence of a protein alone is not proof of its functional role in tumor growth, *in situ* studies by immunohistochemistry in large tumor samples may be valuable in targeting possible autocrine and paracrine loops which merit validation by further functional studies (25), and in correlating the tumor expression level of target proteins with survival.

High stromal VEGF-C expression emerges as an independent protective factor against NSCLC-related death, but the underlying mechanisms remain debatable. Why does an increased expression of angiogenic ligands and receptors in stroma correlate with a better prognosis? It has been shown that tumor cells could change the stromal host compartment and modulate the metabolism and response of resident cells, thus

Table 2. Tumor cell and stromal angiogenic markers as predictors for DSS in 335 patients with NSCLC (univariate analysis; log-rank test)

Marker expression	Patients (*n*)	Patients (%)	Median survival (mo)	5-y survival (%)	*P*
VEGFR-1					
Tumor					
Low	249	74	NR	62	0.013
High	86	26	43	46	
Stroma					
Low	202	60	75	52	0.010
High	133	40	127	64	
VEGFR-2					
Tumor					
Low	140	42	NR	66	0.006
High	195	58	71	51	
Stroma					
Low	268	80	83	54	0.019
High	67	20	127	68	
VEGFR-3					
Tumor					
Low	211	63	NR	65	0.0003
High	120	36	47	45	
Missing	4	1			
Stroma					
Low	225	67	104	56	0.83
High	108	32	127	59	
Missing	2	1			
VEGF-A					
Tumor					
Low	192	57	NR	66	0.0005
High	142	43	52	48	
Missing	1	0			
Stroma					
Low	292	87	83	55	0.017
High	43	13	127	72	
VEGF-C					
Tumor					
Low	231	69	NR	60	0.14
High	104	31	71	52	
Stroma					
Low	266	79	71	53	0.003
High	69	21	127	75	
VEGF-D					
Tumor					
Low	106	32	127	63	0.34
High	228	68	83	55	
Missing	1	0			
Stroma					
Low	237	71	71	53	0.009
High	98	29	127	68	
CD34					
Tumor					
Low	248	74	127	59	0.96
High	80	24	84	55	
Missing	7	2			
Stroma					
Low	264	79	104	56	0.19
High	63	19	127	68	
Missing	8	2			

Abbreviation: NR, not reached.

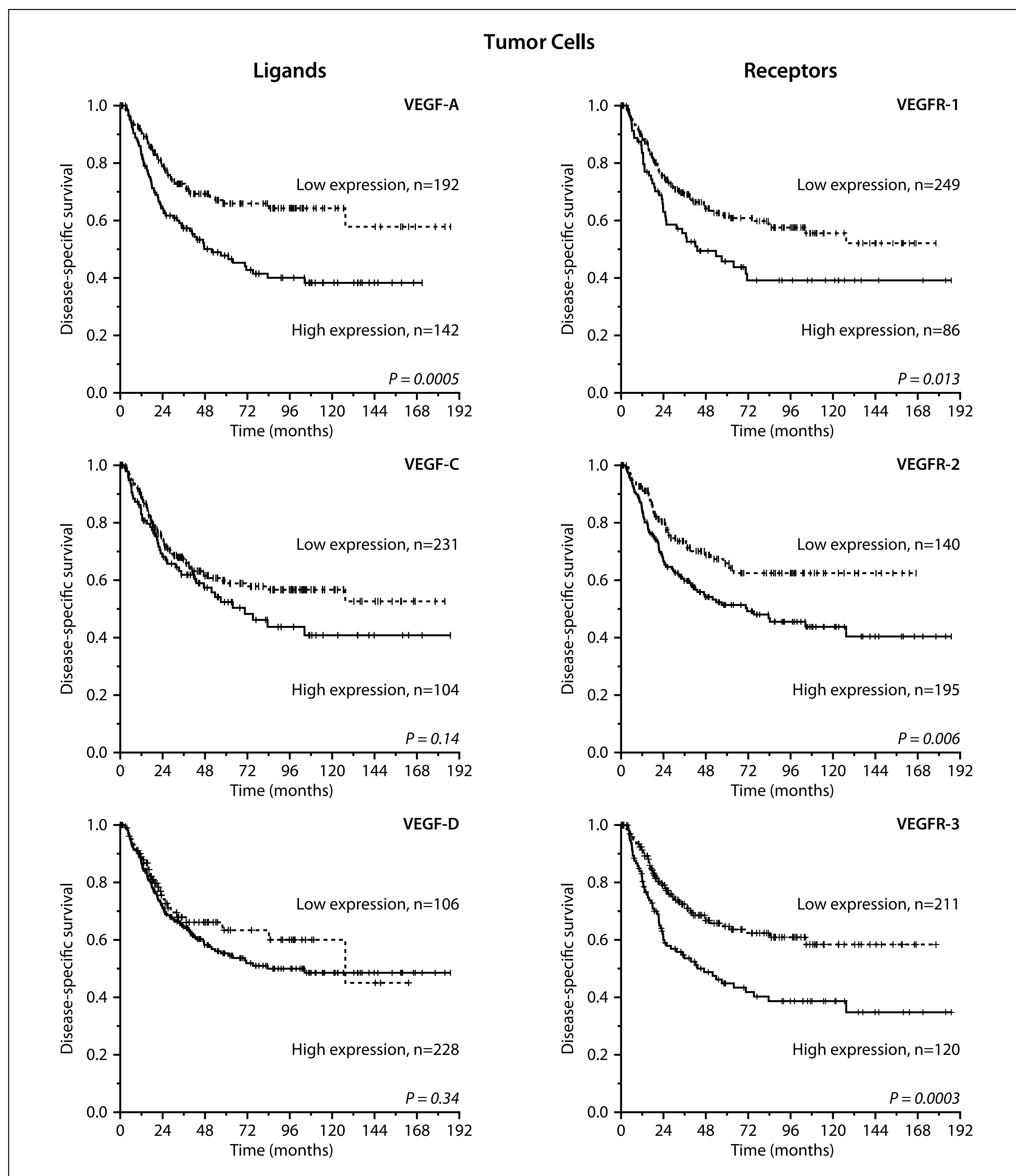

Fig. 2. DSS curves according to VEGF-A, -C, and -D and VEGFR-1, -2 and -3 and expression in tumor cells.

resulting in the formation of a stroma supporting the tumor cells (26). Based on the results presented herein, one may speculate if tissue defense or other mechanisms have the capacity to reverse this process.

Even though proteolytically processed VEGF-C activate VEGFR-2, unlike VEGF-A, the expression of VEGF-C does not seem to be regulated by hypoxia, but is increased by proinflammatory cytokines, indicating a role in inflammatory

responses (27). The immune system has a paradoxical role during cancer development; activation of the adoptive immune system could eradicate malignant cells, whereas activation of various types of innate immune cells may promote tumor development. In a small study including 48 resected NSCLC tumors, Kataki et al. (28) found that one-third of the inflammatory cells in the tumor stroma were macrophages, whereas two-thirds were tumor-infiltrating lymphocytes.

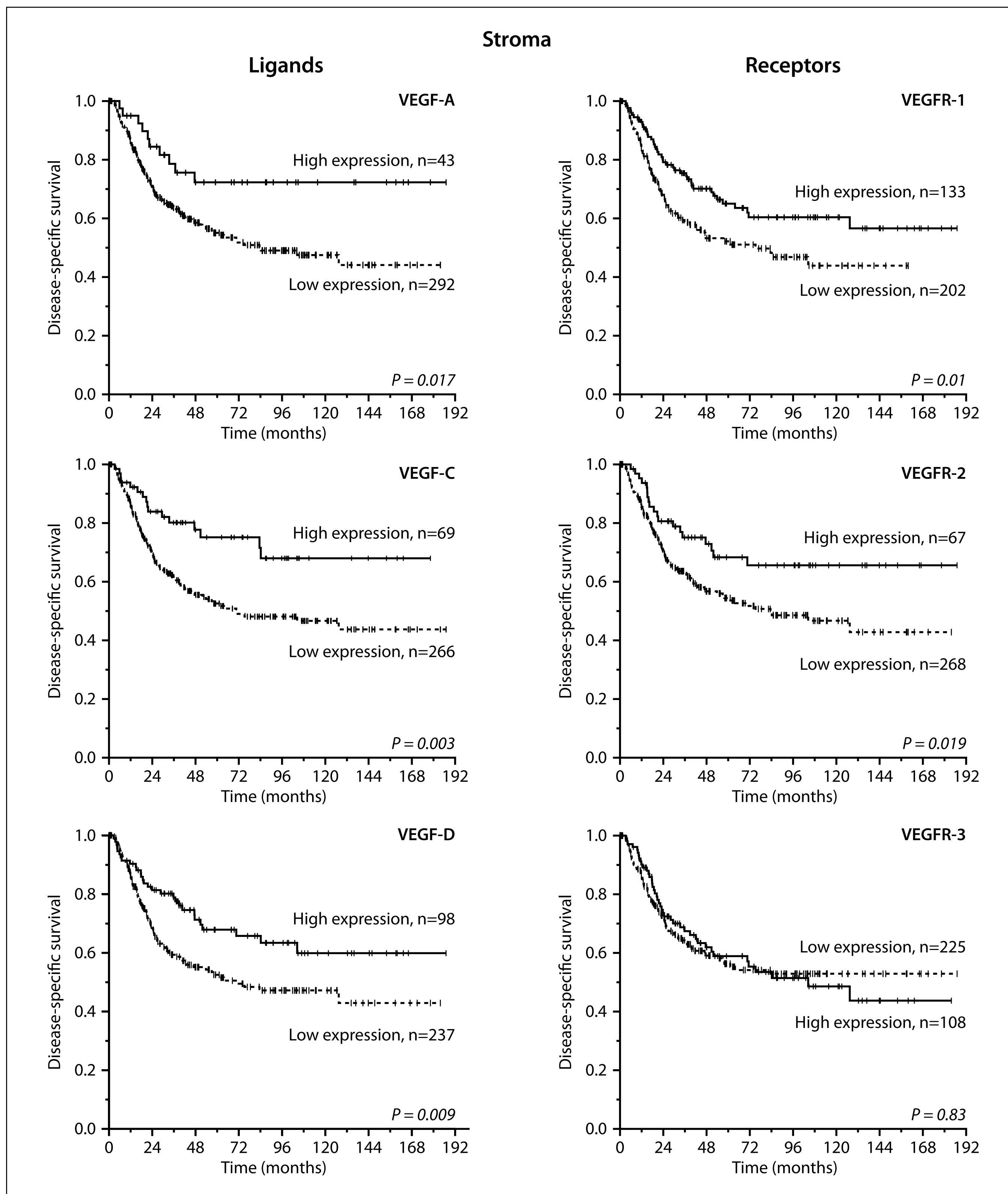

Fig. 3. DSS with respect to VEGF-A, -C, and -D and VEGFR-1, -2, and -3 in stroma.

Table 3. Results of Cox regression analysis summarizing significant independent prognostic factors

Factor	Hazard ratio (95% confidence interval)	*P*
Tumor status		0.003*
1	1.000	
2	1.992 (1.223-3.244)	0.006
3	3.070 (1.520-6.201)	0.002
Nodal status		0.003*
0	1.000	
1	1.889 (1.240-2.878)	0.003
2	1.988 (1.139-3.470)	0.016
Performance status		0.005*
Normal	1.000	
Slightly reduced	1,906 (1.288-2.822)	0.001
In bed <50%	1,648 (0.700-3.882)	0.253
Differentiation		0.018*
Poor	1.000	
Moderate	0.561 (0.373-0.843)	0.005
Well	0.679 (0.375-1.229)	0.201
Vascular infiltration		0.042
No	1.000	
Yes	1.644 (1.019-2.654)	
VEGFR-3 tumor		0.007
Low	1.000	
High	1.686 (1.153-2.466)	
VEGF-C stroma		0.004
Low	2,282 (1.307-3.985)	
High	1.000	

*Overall significance as a prognostic factor.

Whether ligands and receptors might have different functions in stroma versus tumor cells or whether the expression might reflect the density of the adoptive immune system which may protect against tumor development, remains unresolved.

The association between stromal angiogenic factor expression and survival in NSCLC has, to our knowledge, been examined in only two previous studies (29, 30). Ogawa et al. (30) investigated the prognostic impact of stromal VEGF-C in stage I to IIIA NSCLC. Here, VEGF-C was examined in stromal macrophages only, but without demonstrating any prognostic impact. In the present study, the stromal investigation was more comprehensive as macrophages, lymphocytes, granulocytes, plasma cells, and fibroblast-like cells were included in the assessments. The stromal expression presented for each specific VEGF or VEGFR is the total expression following the addition of all stromal cells.

With respect to tumor cells, our results on angiogenic marker expression are consistent with previous findings in studies using conventional tissue sections. The significant correlation between high tumor cell expression of VEGF-A and VEGFR-2 and poor prognosis confirms previous studies (31 – 33). A poor prognostic impact was not seen for tumor cell VEGF-C expression. Although, hitherto, there is no consensus about the prognostic impact of tumor cell VEGF-C expression in NSCLC. Ogawa et al. (30) reported VEGF-C to be an independent prognostic indicator for survival in NSCLC, whereas others detected a significant association in univariate analysis only (34, 35) or not at all (36).

To our knowledge, no previous study has examined the prognostic impact of VEGF-D with regard to DSS in NSCLC. Although high tumor cell expression of VEGF-D has been linked to poor prognosis in gastric (37), colorectal (38), breast (39), ovarian (40), and endometrial carcinomas (41), this association could not be identified in our patients with NSCLC. In contrast, its main receptor, VEGFR-3, was the only significant independent prognostic marker, supporting previously reported findings from a smaller NSCLC study by Arinaga et al. (42).

The interplay between tumor cells and stroma, including the vasculature, is complex, and antiangiogenic therapy has thus far mainly been designed to target the endothelial cells in the vasculature. Interestingly, the VEGFRs are also expressed on cancer cells (43, 44), suggesting the possibility of both autocrine and paracrine growth in VEGF-producing cells (45, 46). This may indicate an additional direct effect of angiogenic inhibitors against tumor cell proliferation. However, because tumor cell targets are often elusive due to mutations, most cells in the tumor stroma are genetically more stable (47) and may accordingly be of greater interest for targeted therapies.

Earlier studies have shown a negative prognostic impact of high MVD in NSCLC (31). Although most studies investigating angiogenesis in tumors have determined MVD by estimating the number of microvessels in the most vascular areas (so-called "hotspots") as described by Weidner, or applied the Chalkley counting technique (48, 49). To our knowledge, this is the first TMA study to evaluate the effect of MVD in NSCLC. We found a significant correlation between high stromal VEGF-C and VEGF-D expression and high MVD. However, these results might be questioned because there was no prognostic impact of MVD in tumor or stromal cores. Neither did we find a correlation between tumor cell VEGF-A expression and MVD. This may be due to the TMA technique's unsuitability for evaluating MVD in NSCLC, consistent with the conclusion after using conventional tissue sections versus TMA in a study evaluating MVD in primary hepatocellular carcinomas (50).

In conclusion, high stromal expression of VEGF-A, -C, -D and VEGFR-1 and -2 favors a good prognosis, with stromal VEGF-C as the only independent positive predictor of survival. The underlying mechanism behind the beneficial role of these angiogenic markers in stroma is not fully understood. Although the TMA technique is suitable for evaluating the role of tumor cell or stromal expression of angiogenic markers, the method seems unsuitable for evaluating MVD in NSCLC. To further elucidate the prognostic role of the stroma in tumor angiogenesis, and its possible role in targeted therapy, additional studies are needed to assess the individual contribution by the different categories of stromal cells.

References

1. Herbst RS, Sandler AB. Non-small cell lung cancer and antiangiogenic therapy: what can be expected of bevacizumab? Oncologist 2004;9 Suppl 1:19 – 26.
2. Yano S, Matsumori Y, Ikuta K, Ogino H, Doljinsuren T, Sone S. Current status and perspective of angiogenesis and antivascular therapeutic strategy: non-small cell lung cancer. Int J Clin Oncol 2006;11:73 – 81.
3. Devesa SS, Blot WJ, Stone BJ, Miller BA, Tarone RE, Fraumeni JF, Jr. Recent cancer trends in the United States. J Natl Cancer Inst 1995;87:175 – 82.
4. Mountain CF. Revisions in the International System for Staging Lung Cancer. Chest 1997;111:1710 – 7.

5. Gasparini G, Longo R, Fanelli M, Teicher BA. Combination of antiangiogenic therapy with other anticancer therapies: results, challenges, and open questions. J Clin Oncol 2005;23:1295–311.

6. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

7. Herbst RS, Onn A, Sandler A. Angiogenesis and lung cancer: prognostic and therapeutic implications. J Clin Oncol 2005;23:3243–56.

8. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–76.

9. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005;23:1011–27.

10. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 1991;5:1806–14.

11. de VC, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992;255:989–91.

12. Millauer B, Wizigmann-Voos S, Schnurch H, et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 1993;72:835–46.

13. Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K. Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. Am J Pathol 2000;156:1499–504.

14. Achen MG, Jeltsch M, Kukk E, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A 1998;95:548–53.

15. Joukov V, Sorsa T, Kumar V, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 1997;16:3898–911.

16. Dumont DJ, Jussila L, Taipale J, et al. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 1998;282:946–9.

17. Su JL, Yang PC, Shih JY, et al. The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. Cancer Cell 2006;9:209–23.

18. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature 2005;438:967–74.

19. Bremnes RM, Camps C, Sirera R. Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. Lung Cancer 2006;51:143–58.

20. Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;438:932–6.

21. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature 2001;411:375–9.

22. World Health Organization. Histological typing of lung and pleural tumours. 3rd ed. Geneva (Switzerland): Springer-Verlag; 1999.

23. Bremnes RM, Veve R, Gabrielson E, et al. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. J Clin Oncol 2002;20:2417–28.

24. Kallioniemi OP, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. Hum Mol Genet 2001;10:657–62.

25. Decaussin M, Sartelet H, Robert C, et al. Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-1 and VEGF-R2-1/KDR) in non-small cell lung carcinomas (NSCLCs): correlation with angiogenesis and survival. J Pathol 1999;188:369–77.

26. De WO, Mareel M. Role of tissue stroma in cancer cell invasion. J Pathol 2003;200:429–47.

27. Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K. Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2002;2:573–83.

28. Kataki A, Scheid P, Piet M, et al. Tumor infiltrating lymphocytes and macrophages have a potential dual role in lung cancer by supporting both host-defense and tumor progression. J Lab Clin Med 2002;140:320–8.

29. Guddo F, Fontanini G, Reina C, Vignola AM, Angeletti A, Bonsignore G. The expression of basic fibroblast growth factor (bFGF) in tumor-associated stromal cells and vessels is inversely correlated with non-small cell lung cancer progression. Hum Pathol 1999;30:788–94.

30. Ogawa E, Takenaka K, Yanagihara K, et al. Clinical significance of VEGF-C status in tumour cells and stromal macrophages in non-small cell lung cancer patients. Br J Cancer 2004;91:498–503.

31. Koukourakis MI, Giatromanolaki A, Thorpe PE, et al. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. Cancer Res 2000;60:3088–95.

32. O'Byrne KJ, Koukourakis MI, Giatromanolaki A, et al. Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small-cell lung cancer. Br J Cancer 2000;82:1427–32.

33. Volm M, Koomagi R, Mattern J. Interrelationships between microvessel density, expression of VEGF and resistance to doxorubicin of non-small lung cell carcinoma. Anticancer Res 1996;16:213–7.

34. Kajita T, Ohta Y, Kimura K, et al. The expression of vascular endothelial growth factor C and its receptors in non-small cell lung cancer. Br J Cancer 2001;85:255–60.

35. Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S. Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. Clin Cancer Res 2000;6:2431–9.

36. Ohta Y, Nozawa H, Tanaka Y, Oda M, Watanabe Y. Increased vascular endothelial growth factor and vascular endothelial growth factor-c and decreased nm23 expression associated with microdissemination in the lymph nodes in stage I non-small cell lung cancer. J Thorac Cardiovasc Surg 2000;119:804–13.

37. Juttner S, Wissmann C, Jons T, et al. Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. J Clin Oncol 2006;24:228–40.

38. White JD, Hewett PW, Kosuge D, et al. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. Cancer Res 2002;62:1669–75.

39. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Flt-4-positive vessel density correlates with vascular endothelial growth factor-d expression, nodal status, and prognosis in breast cancer. Clin Cancer Res 2003;9:5313–7.

40. Yokoyama Y, Charnock-Jones DS, Licence D, et al. Vascular endothelial growth factor-D is an independent prognostic factor in epithelial ovarian carcinoma. Br J Cancer 2003;88:237–44.

41. Yokoyama Y, Charnock-Jones DS, Licence D, et al. Expression of vascular endothelial growth factor (VEGF)-D and its receptor, VEGF receptor 3, as a prognostic factor in endometrial carcinoma. Clin Cancer Res 2003;9:1361–9.

42. Arinaga M, Noguchi T, Takeno S, Chujo M, Miura T, Uchida Y. Clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in patients with nonsmall cell lung carcinoma. Cancer 2003;97:457–64.

43. Dziadziuszko R, Chyczewski L, Jassem E, Jassem J. Expression of vascular endothelial growth factor (VEGF) and its receptor FLK-1 in non-small cell lung cancer (NSCLC)—a preliminary report. Folia Histochem Cytobiol 2001;39:100–1.

44. Zhukova LG, Zhukov NV, Lichinitser MR. Expression of Flt-1 and Flk-1 receptors for vascular endothelial growth factor on tumor cells as a new prognostic criterion for locally advanced breast cancer. Bull Exp Biol Med 2003;135:478–81.

45. Masood R, Cai J, Zheng T, Smith DL, Hinton DR, Gill PS. Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. Blood 2001;98:1904–13.

46. Strizzi L, Catalano A, Vianale G, et al. Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma. J Pathol 2001;193:468–75.

47. Loeffler M, Kruger JA, Niethammer AG, Reisfeld RA. Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. J Clin Invest 2006;116:1955–62.

48. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. N Engl J Med 1991;324:1–8.

49. Fox SB, Leek RD, Weekes MP, Whitehouse RM, Gatter KC, Harris AL. Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. J Pathol 1995;177:275–83.

50. Yao Y, Pan Y, Chen J, Sun X, Qiu Y, Ding Y. Endoglin (CD105) expression in angiogenesis of primary hepatocellular carcinomas: analysis using tissue microarrays and comparisons with CD34 and VEGF. Ann Clin Lab Sci 2007;37:39–48.