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The clinical significance of lymphangiogenesis and angiogenesis in non-small cell lung cancer patients

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ABSTRACT

Background: Angiogenesis and lymphangiogenesis have been reported to affect malignant phenotype.
Method: We investigated 147 patients with non-small cell lung cancer (NSCLC). Immunohistochemistry using D2-40 was performed to evaluate lymphatic vessel density (LVD), including Micro-LVD (without lumen), Tubal-LVD (with lumen) and lymphatic vessel invasion (LVI). The intratumoural microvessel density (MVD) was evaluated by CD-34 immunostaining. The expressions of vascular endothelial growth factor-A (VEGF-A) and VEGF-C were also studied.
Results: Lymphangiogenesis was significantly associated with Micro-LVD ($p = 0.0003$). The VEGF-C expression was significantly associated with the Micro-LVD ($p = 0.0057$). In contrast, the VEGF-A expression was significantly associated with the MVD ($p = 0.0092$). The survival was significantly lower in patients with Micro-LVD-high tumours than in patients with Micro-LVD-low tumours ($p = 0.0397$). Survival was also significantly lower in patients with MVD-high tumours than in patients with MVD-low tumours ($p = 0.0334$). A multivariate analysis demonstrated that the Micro-LVD ($p = 0.0363$) and the MVD ($p = 0.0232$) were independent prognostic factors for NSCLC patients.
Conclusions: Lymphangiogenesis, specifically Micro-LVD and angiogenesis are independently associated with a poor prognosis in NSCLC patients.

1. Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers and the major cause of cancer-related death in North America, Europe and Japan.[1] In this situation, understanding the various biologic behaviours of NSCLCs, including tumour growth and metastatic potential, is important for improving the treatment of NSCLCs.[2] Recent advances in molecular biology have raised the possibilities of new treatments for NSCLCs, such as tailor-maid chemotherapy based on biomarkers or molecular-target therapy.[3]

Amongst various types of tumour activities, angiogenesis and lymphangiogenesis have been reported to affect malignant phenotype, such as metastasis. It is widely accepted that

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angiogenesis is essential for both tumour growth and metastasis.[4,5] In fact, many clinical studies have demonstrated that angiogenesis is associated with tumour metastasis and a poor prognosis in cancer patients.[6,7] On the other hand, lymphangiogenesis has also been reported to be involved in lymphatic metastasis.[5] Lymphatic spread of the tumour is assumed to occur by cancer cell permeation of intratumourous lymphatics, therefore reaching the regional lymph nodes. However, lymphangiogenesis has been difficult to investigate until recent years because there was a lack of specific monoclonal antibodies selectively recognising the lymphatic endothelium. Recently, a monoclonal antibody D2-40, a selective marker for lymphatic endothelium,[8,9] has been developed for clinical studies on lymphangiogenesis in human cancers, including NSCLCs.[10–12]

Understanding the precise mechanisms of lymphangiogenesis could lead to the development of new treatments for NSCLC patients. In addition, there have been only a few clinical reports which made a comprehensive evaluation of lymphangiogenesis and angiogenesis in NSCLCs. In order to clarify their clinical significance in NSCLCs, a clinical study was conducted on lymphangiogenesis, including a morphological analysis and angiogenesis. Furthermore, amongst various biomarkers, previous experimental and clinical studies have demonstrated that vascular endothelial growth factor (VEGF) family plays roles in the physiological and pathological regulation of angiogenesis and lymphangiogenesis.[13] Therefore, additional evaluations of the intratumoural expression of VEGF-A and VEGF-C were performed.

2. Materials and methods

2.1. Clinical characteristics of the patients

Consecutive NSCLC patients who underwent surgery at the Faculty of Medicine, Kagawa University from January 1998 to June 2002 were studied. This study was approved by the institutional review board of Kagawa University (14-7, a clinical study of biologic markers in NSCLCs) and signed informed consent was obtained from each patient. Tumour-node-metastasis (TNM) staging designations were made according to the postsurgical pathological international staging system. Because advanced stage lung cancer (stage IV) involved several ill-defined factors and had distant metastases, such patients were excluded from this study. In total, 147 patients with lung cancer up to stage III, including 93 patients with adenocarcinomas, 49 patients with squamous cell carcinomas and 5 patients with large cell carcinomas, were investigated (Table 1). Patients' clinical records and histopathological diagnoses were fully documented. This report includes the follow-up data as of 31st October 2006.

With regard to the methods of surgical resection, a pneumonectomy was done in 13 patients with stage II–III NSCLC. A lobectomy was done in 119 patients: 74 patients with stage I NSCLC, 15 patients with stage II NSCLC and 30 patients with stage III NSCLC. A segmentectomy was done in four patients with stage I NSCLC and a wedge resection was done in 11 patients with stage I NSCLC. Systemic chemotherapy using mitomycin/vinblastin/cisplatin or carboplatin/paclitaxel was done in all the patients with stage II–III NSCLC: neoadjuvant chemotherapy in 37 patients and postoperative adjuvant chemotherapy in 21 patients with nodal metastases. Radiation therapy was done in 21 patients: 11 patients with T3 or T4 status and 10 patients with mediastinal lymph node metastases.

Table 1 – Demographic and clinical characteristics of patients

Characteristics	Number of patients	Percent
Total number of patients	147	100
Age		
Median	67	
Range	35–82	
Gender		
Male	100	68.0
Female	47	32.0
Smoking status		
Non-smoker	47	32.0
Smoker	100	68.0
ECOG performance status		
0	78	53.1
1	55	37.4
2	14	9.5
Pathological stage		
Stage I	89	60.6
Stage II	19	12.9
Stage III	39	26.5
Histology		
Adenocarcinoma	93	63.3
Squamous cell carcinoma	49	33.3
Large cell carcinoma	5	3.4
Method of surgical resection		
Pneumonectomy	13	8.8
Lobectomy	119	81.0
Segmentectomy	4	2.7
Wedge resection	11	7.5
Chemotherepy	58	39.5
Neoadjuvant chemotherapy	37	25.2
Postoperative adjuvant chemotherapy	21	14.3
Radiotherapy	21	14.3

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

2.2. Immunohistochemistry

We used a mouse monoclonal antibody for D2-40 (DakoCytomation, Glostrup, Denmark) diluted at 1:100, a mouse monoclonal antibody for CD34 (NU-4A1, Nichirei Corporation, Tokyo, Japan) diluted at 1:10, a goat polyclonal antibody for VEGF-C (N-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted at 1:100 and a rabbit polyclonal antibody for VEGF-A (A-20, Santa Cruz) diluted at 1:200. Formalin-fixed paraffin-embedded tissue was cut in 4 μm sections and mounted on poly-L-lysine-coated slides. Sections were deparaffinised and rehydrated. The slides were then heated in a microwave for 10 min in a 10 μmol/L citrate buffer solution at pH 6.0, and cooled to room temperature. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in abso-

lute methanol) for 30 min, the sections were treated for 2 h at room temperature with 5% bovine serum albumin. Duplicate sections were then incubated overnight with the primary specific antibodies against D2-40, CD34, VEGF-C and VEGF-A, respectively. The slides were then incubated for 1 h with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) for D2-40 and CD34, biotinylated anti-goat IgG (Vector) for VEGF-C and biotinylated anti-rabbit IgG (Vector) for VEGF-A. The sections were incubated with the avidin–biotin-peroxidase complex (Vector) for 1 h, and antibody binding was visualised with 3,3′-diaminobenzidine tetrahydrochloride. Lastly, the sections were lightly counterstained with Mayer's haematoxylin. Sections of resected lung tumours known to express VEGF-C or VEGF-A were used as positive controls for immunohistochemical staining. Sections incubated with normal goat IgG served as negative reaction controls for staining of VEGF-C, and sections incubated with normal rabbit IgG served as negative reaction controls for staining of VEGF-A.

All of the immunostained sections were evaluated by two authors (K.K. and M.U.) who had no knowledge of the patients' clinical status. For lymphatic microvessel quantification, the three most highly vascularised areas detected by D2-40 immunostaining were initially selected under the ×40 field, and a ×200 field (0.785 mm^2 per field) was used to count D2-40-positive vessels in each of these areas. Lymphatic vessels were classified into three categories. The average of three ×200 field counts of D2-40-positive single endothelial cells or the cluster of D2-40-positive endothelial cells without lumen was recorded as the micro-lymphatic vessel density (Micro-LVD). The average of three ×200 field counts of D2-40-positive lymphatic vessels with lumens was recorded as the tubal-lymphatic vessel density (Tubal-LVD). The sum of Micro-LVD and Tubal-LVD was regarded as total lymphatic vessel density (Total-LVD). In addition, lymphatic vessel invasion (LVI) was defined if at least one tumour cell cluster was visible within D2-40-positive lining spaces. Because each cut-off value showed the most significance in relation to the LVI, the tumour was classified as Micro-LVD-high when Micro-LVD $\geqslant$ 15 Tubal-LVD-high when Tubal-LVD $\geqslant$ 15 and Total-LVD-high when Total-LVD $\geqslant$ 30.

For microvessel quantification, the three most highly vascularised areas detected by CD34 immunostaining were also selected under the ×40 field. The average of three ×200 field counts of CD34-positive vessels was recorded as the intratumoural microvessel density (MVD). Tumours with MVD $\geqslant$ 90 were classified as MVD-high, based on the findings of the previous reports.[2,7]

For the VEGF-C and VEGF-A expression status, the proportion of staining tumour cells in each selected field was determined by counting individual tumour cells at high magnification. In the cases with multiple areas of low intensity that occurred during the evaluation of immunostaining, five areas were selected at random and scored. One random field was selected in sections where all staining appeared intense. At least 200 tumour cells were scored per ×40 field. Samples were classified into two groups, positive and negative, with a cut-off value based on the findings of the previous reports.[2] When $\geqslant$30% of the carcinoma cells in a given specimen were positively stained for VEGF-C, the sample was classified as VEGF-C-positive.[2,10] When $\geqslant$30% of the carcinoma cells in a given specimen were positively stained for VEGF-A, the sample was classified as VEGF-A-positive.[2,7]

2.3. Statistical analysis

Because the distributions of the Micro-LVD ($p < 0.001$) the Tubal-LVD ($p < 0.001$) and Total-LVD ($p = 0.014$) showed no normal distributions (Kolmogorov–Smirnov analysis), the statistical differences regarding these variables in relation to several clinical and pathological parameters were assessed using the Mann–Whitney's *U*-test. Because the distributions of the MVD showed normal distributions ($p = 0.280$, Kolmogorov–Smirnov analysis), the statistical differences regarding these variables in relation to several clinical and pathological parameters were assessed by the Student's *t*-test, and an analysis of variance with the Bonferroni/Dunn test. The overall survival was defined as the time from the treatment initiation (surgical resection, chemotherapy or radiation) to the date of death from any cause. The Kaplan–Meier method was used to estimate the probability of overall survival as a function of time, and differences in the survival of subgroups of patients were compared by using Mantel's log-rank test. A multivariate analysis was performed using the Cox regression model to study the effects of different variables on survival. All *p* values were based on two-tailed statistical analysis, and a *p* value <0.05 was considered to indicate statistical significance.

3. Results

3.1. Lymphangiogenesis in NSCLCs

In lymphatic endothelial cells without lumens, the Micro-LVD ranged from 0 to 92 (median, 12; mean ± SD, 24.6 ± 25.9, Fig. 1A). Of the 147 NSCLCs studied, 65 carcinomas (44.2%) were Micro-LVD-high (Table 2). Regarding tumour histology, the Micro-LVD was significantly higher in squamous cell carcinomas than in adenocarcinomas (39.1 ± 31.3 versus 15.6 ± 17.1, $p < 0.0001$, Table 2). For tumour differentiation, the Micro-LVD was 14.9 ± 19.3 in well-differentiated tumours, 24.2 ± 25.1 in moderately differentiated tumours and 38.2 ± 29.0 in poorly differentiated tumours. The Micro-LVD was significantly lower in well-differentiated tumours in comparison to moderately differentiated and poorly differentiated tumours ($p = 0.0002$). The Micro-LVD was also significantly associated with tumour status ($p = 0.0020$). Furthermore, the Micro-LVD was significantly higher in node-positive tumours than in node-negative tumours (32.7 ± 31.8 versus 21.4 ± 22.6, $p = 0.0343$).

In lymphatic vessels with lumens, the Tubal-LVD ranged from 3 to 90 (median, 13; mean ± SD, 18.4 ± 15.8, Table 2). However, no difference was observed in the Tubal-LVD according to tumour histology, tumour differentiation and pathological stage (Table 1).

In total, the Total-LVD ranged from 3 to 150 (median, 33; mean ± SD, 42.9 ± 33.4, Table 2). The Total-LVD was significantly higher in squamous cell carcinomas than in adenocarcinomas (56.9 ± 36.7 versus 34.4 ± 28.7, $p = 0.0005$). Regarding tumour differentiation, the Total-LVD was significantly higher

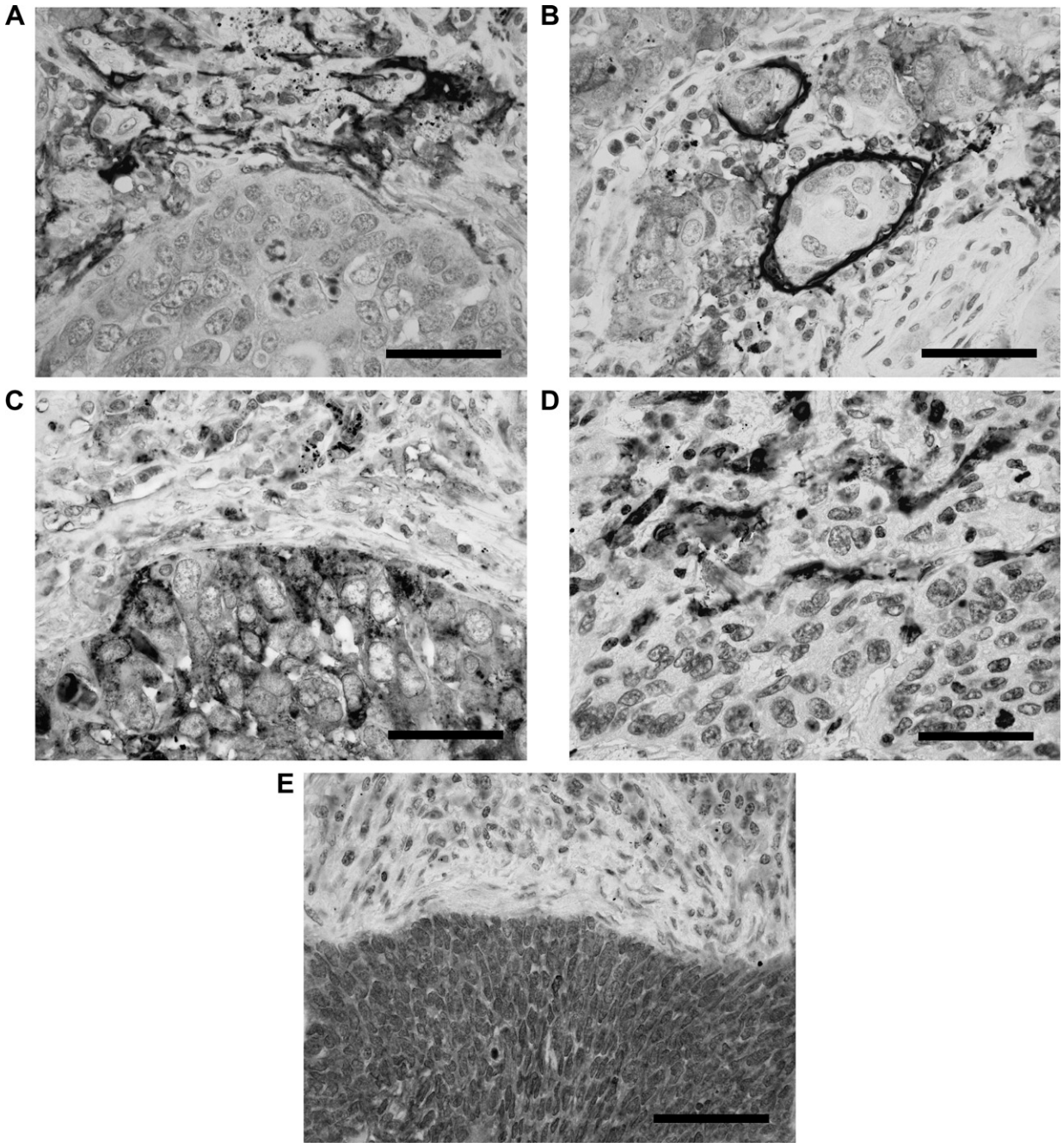

Fig. 1 – Immunohistochemical staining of human non-small cell lung cancer tissues using the avidin-biotin-peroxidase complex procedure. A Micro-LVD-high carcinoma (A) with lymphatic vessel invasion (B) and positive expression of VEGF-C (C). A MVD-high carcinoma (D) with positive expression of VEGF-A (E). Bar, 50 μm.

in poorly differentiated tumours in comparison to moderately differentiated and well-differentiated tumours ($p = 0.0306$). In addition, the Total-LVD was significantly associated with tumour status ($p = 0.0025$).

3.2. *Lymphatic vessel invasion of NSCLCs in relation to lymphangiogenesis*

To investigate the clinical significance of lymphangiogenesis of NSCLCs, the lymphatic vessel invasion (LVI) was evaluated (Fig. 1B). Of the 147 NSCLCs studied, 49 tumours (33.3%) had LVI. Regarding nodal status, of the 49 tumours with LVI, 20 tumours (40.8%) were node-positive. Of the 98 tumours without LVI, 21 tumours (21.4%) were node-positive. The LVI was significantly associated with nodal status ($p = 0.0135$). Furthermore, the LVI was also significantly associated with the pathological stage ($p = 0.0064$).

Of the 65 Micro-LVD-high tumours, 32 tumours (49.2%) had LVI. Of the 82 Micro-LVD-low tumours, 17 tumours (20.7%) had LVI. The ratio of LVI-positive tumours was significantly higher

Table 2 – Distributions of lymphatic vessels in 147 NSCLC patients

Characteristics	*n*	Micro-LVD			Tubal- LVD			Total-LVD		
			p-Value	High (%)		*p*-Value	High (%)		*p*-Value	High (%)
Age										
<60	35	21.9 ± 24.6	0.4911	37.1	17.2 ± 14.6	0.6249	42.9	39.1 ± 35.0	0.4437	37.1
⩾60	112	25.4 ± 26.4		46.4	18.7 ± 16.2		46.4	44.1 ± 32.9		50.0
Gender										
Male	100	25.5 ± 25.8	0.5353	46.0	17.6 ± 12.6	0.3700	46.0	43.0 ± 33.1	0.9546	47.0
Female	47	22.6 ± 26.3		40.4	20.1 ± 21.1		44.7	42.7 ± 34.3		46.8
Smoking										
Non-smoker	47	15.6 ± 19.8	0.0030	27.6	18.7 ± 21.4	0.8813	36.2	34.2 ± 31.4	0.0127	34.0
Smoker	100	28.8 ± 27.4		52.0	18.2 ± 12.5		50.0	47.0 ± 33.7		53.0
Tumor status										
T1, T2	62	20.6 ± 23.2	0.0020	37.1	17.3 ± 15.8	0.1473	40.7	37.9 ± 30.9	0.0025	43.4
T3, T4	51	37.7 ± 30.3		41.2	21.8 ± 15.6		61.8	59.5 ± 36.5		58.8
Nodal status										
N0	106	21.4 ± 22.6	0.0343	41.5	18.3 ± 16.0	0.4868	43.4	39.7 ± 30.2	0.0840	45.3
N1, N2, N3	41	32.7 ± 31.8		51.2	18.5 ± 15.6		51.2	51.2 ± 39.7		51.2
Pathological stage										
Stage I	89	18.4 ± 20.5	0.0008	37.1	17.5 ± 15.6	0.1514	40.4	35.9 ± 28.4	0.0034	41.6
Stage II	19	37.7 ± 26.4		63.2	22.8 ± 18.7		57.9	60.5 ± 36.5		73.7
Stage III	39	32.3 ± 32.2		51.3	18.2 ± 14.8		51.3	50.4 ± 38.1		46.2
Differentiation										
Well	54	14.9 ± 19.3	0.0002	29.6	21.4 ± 20.5	0.8502	44.4	36.3 ± 32.8	0.0306	35.2
Moderately	53	24.2 ± 25.1		45.3	15.4 ± 11.5		39.6	39.5 ± 32.1		47.2
Poorly	40	38.2 ± 29.0		62.5	18.3 ± 12.7		55.0	56.4 ± 32.8		62.5
Histology										
Adenocarcinoma	93	15.6 ± 17.1	<0.0001[a]	33.3	18.8 ± 18.4	0.0861[a]	39.8	34.4 ± 28.7	0.0005[a]	36.6
Squamous cell carcinoma	49	39.1 ± 31.3		59.2	17.7 ± 10.4		53.1	56.9 ± 36.7		61.2
Large cell carcinoma	5	47.8 ± 29.6		100.0	16.8 ± 5.0		80.0	64.6 ± 30.7		100.0
Total number of patients	147	24.6 ± 25.9		44.2	18.4 ± 15.8		45.6	42.9 ± 33.4		46.9

Abbreviation: LVD, lymphatic vessel density.
a Adenocarcinoma versus squamous cell carcinoma.

in Micro-LVD-high tumours than in Micro-LVD-low tumours ($p = 0.0003$, Fig. 2A). In addition, the Micro-LVD was 34.7 ± 28.4 in LVI-positive tumours, and 19.5 ± 23.1 in LVI-negative tumours. The Micro-LVD was significantly higher in LVI-positive tumours than in LVI-negative tumours ($p = 0.0002$).

The ratio of LVI-positive tumours was also significantly higher in Tubal-LVD-high tumours than in Tubal-LVD-low tumours (47.8% versus 21.3%, $p = 0.0007$, Fig. 2B). In total, the ratio of LVI-positive tumours was significantly higher in Total-LVD-high tumours than in Total-LVD-low tumours (46.4% versus 21.8%, $p = 0.0016$, Fig. 2C). In addition, the Total-LVD was significantly higher in LVI-positive tumours than in LVI-negative tumours (58.3 ± 37.4 versus 35.3 ± 28.4, $p < 0.0001$).

3.3. *The intratumoural VEGF-C expression in NSCLCs*

The intratumoural expression of VEGF-C and VEGF-A was evaluated in relation to lymphangiogenesis and angiogenesis in NSCLCs. Intratumoural VEGF-C staining exhibited a cytoplasmic staining pattern (Fig. 1C). Of the 147 tumours, 65 tumours (44.2%) were VEGF-C-positive. Regarding the lymphangiogenesis, the Micro-LVD was 31.1 ± 28.6 in VEGF-C-positive tumours, and 19.4 ± 22.4 in VEGF-C-negative tumours. The Micro-LVD was significantly higher in VEGF-C-positive tumours than in VEGF-C-negative tumours ($p = 0.0057$, Fig. 3A). However, there was no difference in the Tubal-LVD between VEGF-C-positive tumours and VEGF-C-negative tumours (18.8 ± 15.0 versus 18.1 ± 16.5, Fig. 3B). Regarding the angiogenesis, no difference was observed in the MVD between VEGF-C-positive tumours and VEGF-C-negative tumours (96.8 ± 48.4 versus 104.2 ± 41.5, Fig. 3C).

3.4. *The intratumoural VEGF-A expression in NSCLCs*

Intratumoural VEGF-A staining also exhibited a cytoplasmic staining pattern. Of the 147 tumours, 69 tumours (46.9%) were VEGF-A-positive (Fig. 1E). There was no correlation between the intratumoural VEGF-A expression and the intratumoural

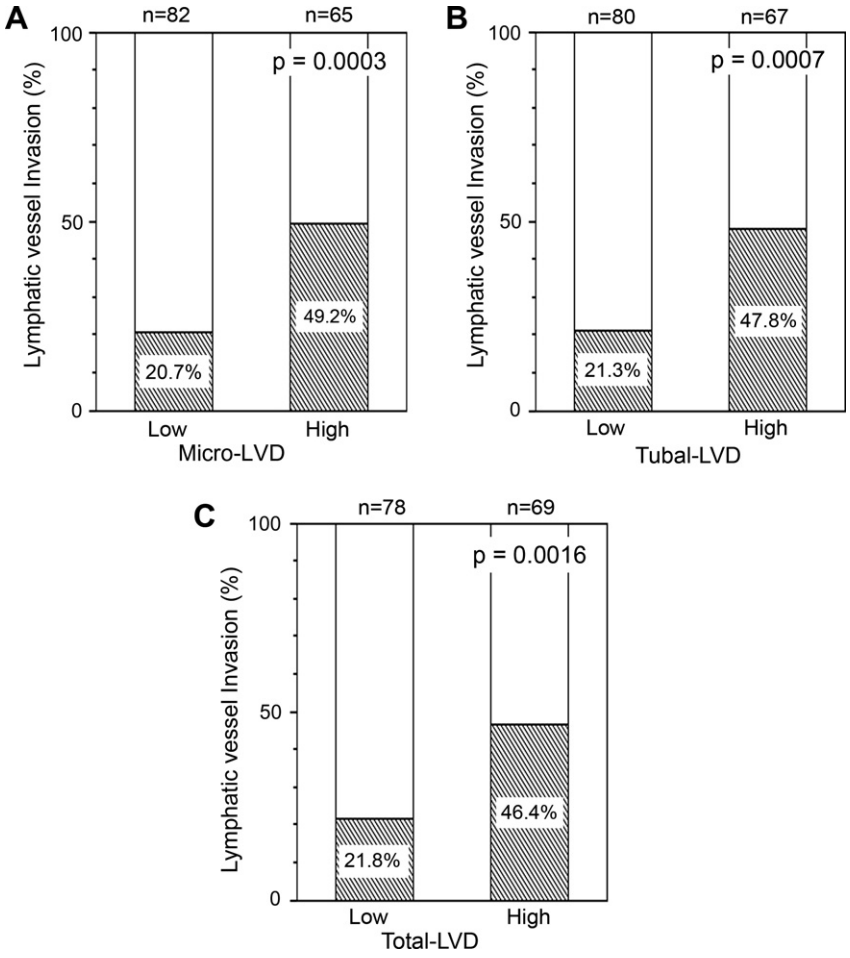

Fig. 2 – (A) Lymphatic vessel invasion in relation to Micro-LVD. (B) Lymphatic vessel invasion in relation to Tubal-LVD. (C) Lymphatic vessel invasion in relation to Total-LVD.

VEGF-C expression ($r = 0.045$, $p = 0.5855$). Regarding the lymphangiogenesis, there was no difference in the Micro-LVD or the Tubal-LVD according to VEGF-A status (Figs. 4A and B). In contrast, the MVD was significantly higher in VEGF-A-positive tumours than in VEGF-A-negative tumours (111.1 ± 49.8 versus 92.0 ± 37.7, $p = 0.0092$, Fig. 4C). The ratio of MVD-high tumours was significantly higher in VEGF-A-positive tumours than in VEGF-A-negative tumours (63.8% versus 37.2%, $p = 0.0013$).

3.5. *Overall survival of NSCLC patients*

Regarding the lymphangiogenesis, the 5-year survival rate was 53.8% in patients with Micro-LVD-high tumours, and 68.0% in patients with Micro-LVD-low tumours. The overall survival was significantly lower in patients with Micro-LVD-high tumours than in patients with Micro-LVD-low tumours ($p = 0.0397$, Fig. 5A). In contrast, no difference was observed in the patient survival according to the Tubal-LVD or the Total-LVD (Figs. 5B and C). The 5-year survival rate was significantly lower in patients with LVI-positive tumours than in patients with LVI-negative tumours (50.8% versus 67.6%, $p = 0.0095$, Fig. 5D). Regarding the angiogenesis, the 5-year survival rate was significantly lower in patients with MVD-high tumours than in patients with MVD-low tumours (49.8% versus 72.1% $p = 0.0334$, Fig. 5E). Moreover, the 5-year survival rate was significantly lower in patients with VEGF-C-positive tumours than in patients with VEGF-C-negative tumours (49.6% versus 70.5%, $p = 0.0299$, Fig. 5F). In addition, the 5-year survival rate was also significantly lower in patients with VEGF-A-positive tumours than in patients with VEGF-A-negative tumours (49.1% versus 70.4%, $p = 0.0332$, Fig. 5G).

Univariate analyses using the Cox regression model also revealed that six variables, pathological stage (hazard ratio 1.562; $p = 0.0030$), Micro-LVD (hazard ratio 1.775; $p = 0.0425$), LVI (hazard ratio 2.040; $p = 0.0111$), MVD (hazard ratio 1.826; $p = 0.0361$), VEGF-C status (hazard ratio 1.830; $p = 0.0324$) and VEGF-A status (hazard ratio 1.815; $p = 0.0358$), were significant prognostic factors for NSCLC patients (Table 3). Furthermore, a multivariate analysis using the Cox regression model demonstrated the Micro-LVD (hazard ratio 1.838; $p = 0.0363$) and the MVD (hazard ratio 1.942; $p = 0.0232$) to be independent prognostic factors for NSCLC patients (Table 4A). Another multivariate analysis revealed that VEGF-C status (hazard ratio 1.801; $p = 0.0410$) and VEGF-A status (hazard ratio 2.006; $p = 0.0152$) were also independent prognostic factors for NSCLC patients (Table 4B).

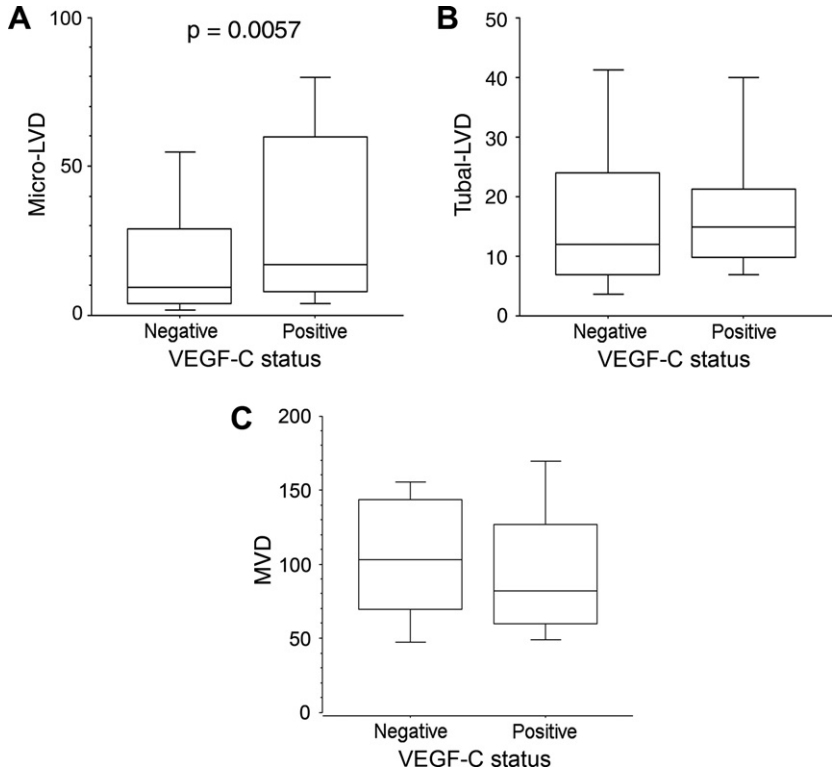

Fig. 3 – (A) Micro-LVD in relation to the VEGF-C status. (B) Tubal-LVD in relation to the VEGF-C status. (C) MVI in relation to the VEGF-C status.

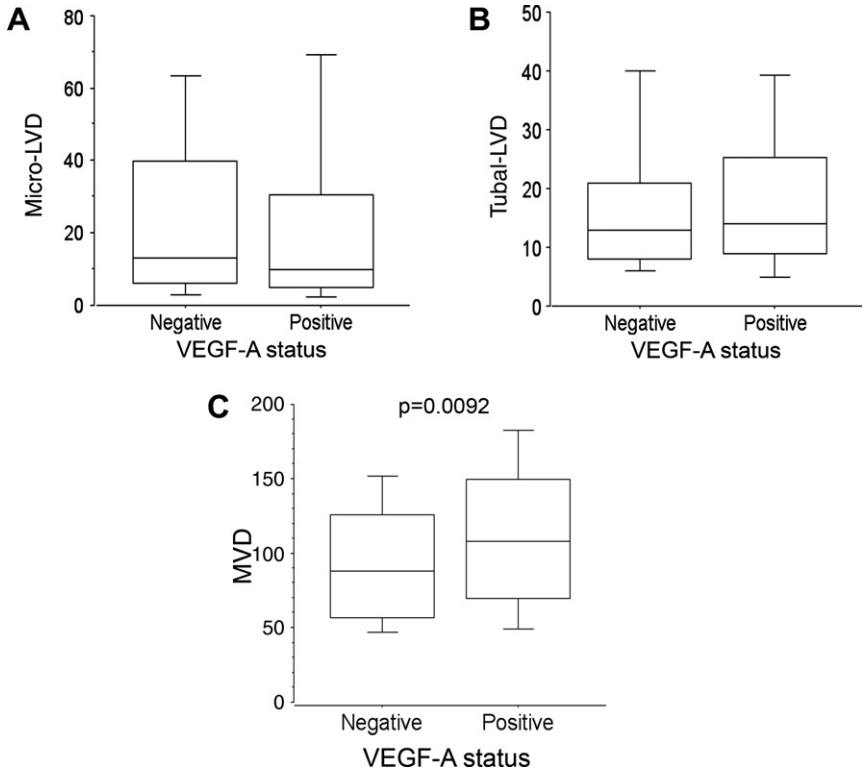

Fig. 4 – (A) Micro-LVD in relation to the VEGF-A status. (B) Tubal-LVD in relation to the VEGF-A status. (C) MVI in relation to the VEGF-A status.

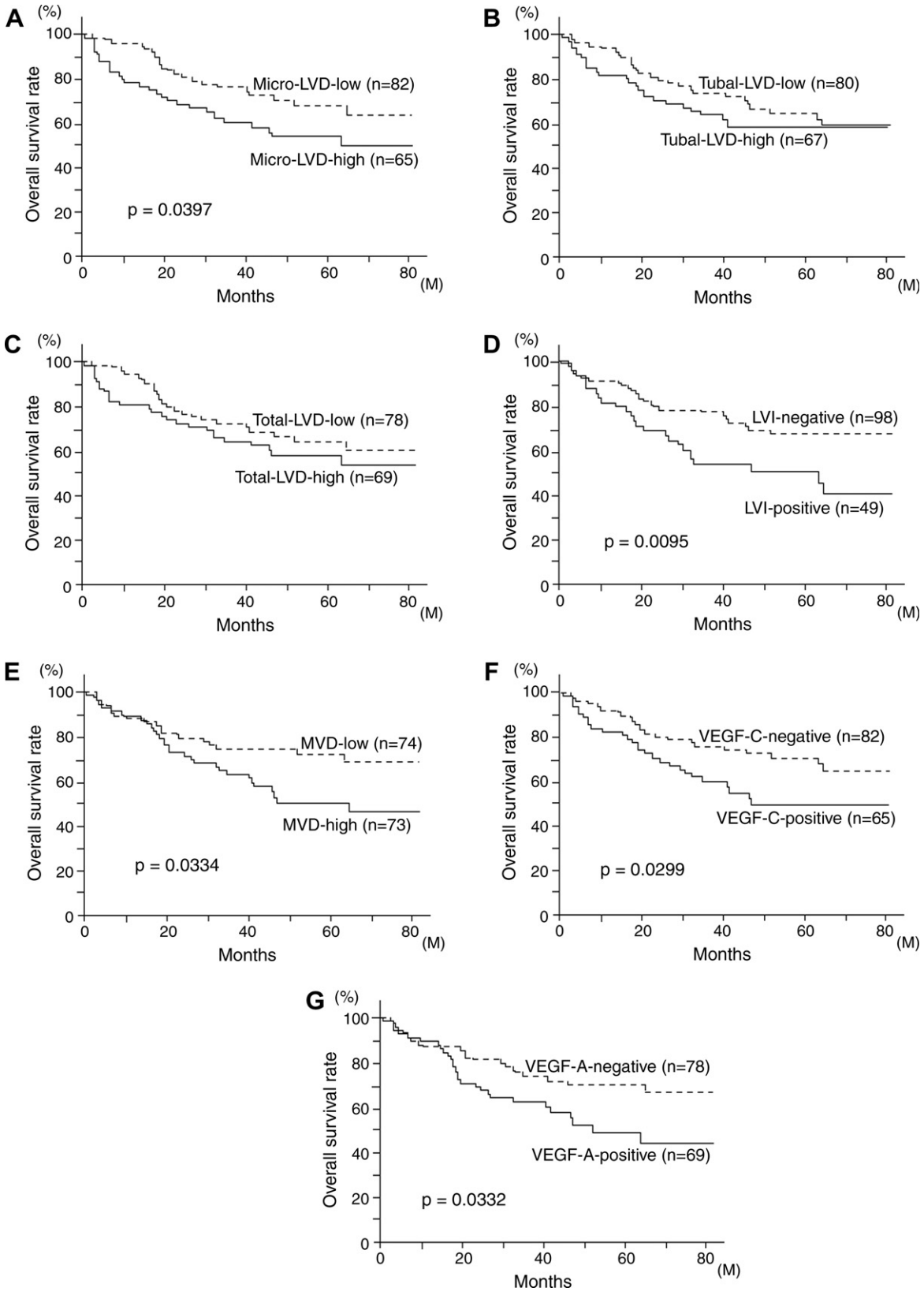

Fig. 5 – Overall survival in relation to Micro-LVD (A), Tubal-LVD (B), Total-LVD (C), lymphatic invasion (D), MVD (E), the VEGF-C status (F) and the VEGF-A status (G).

Table 3 – Univariate regression analysis in predicting the survival of NSCLC patients

Variables	Hazard ratio	95% CI	*p*-Value
Age	0.993	(0.968–1.019)	0.5902
Gender: Male/Female	0.592	(0.315–1.113)	0.1039
Smoking: non-smoker/smoker	1.881	(0.983–3.596)	0.0562
Pathological stage	1.562	(1.164–2.095)	0.0030
Micro-LVD	1.775	(1.020–3.089)	0.0425
LVI	2.040	(1.177–3.536)	0.0111
MVD	1.826	(1.040–3.207)	0.0361
VEGF-C status	1.830	(1.052–3.183)	0.0324
VEGF-A status	1.815	(1.040–3.166)	0.0358

Abbreviations: CI, confidence interval; LVD, lymphatic vessel density; LVI, lymphatic vessel invasion; MVD, microvessel density.

Table 4A – Multivariate regression analysis in predicting the survival of NSCLC patients using Micro-LVD and MVD

Variables	Hazard ratio	95% CI	*p*-Value
Age	1.001	(0.974–1.028)	0.9574
Gender: Male/Female	0.831	(0.319–2.166)	0.7047
Smoking: non-smoker/smoker	1.746	(0.635–4.802)	0.2802
Pathological stage	1.497	(1.093–2.050)	0.0119
Micro-LVD	1.838	(1.039–3.249)	0.0363
MVD	1.942	(1.095–3.443)	0.0232

Table 4B – Multivariate regression analysis in predicting the survival of NSCLC patients using VEGF-C and VEGF-A status

Variables	Hazard ratio	95% CI	*p*-Value
Age	0.998	(0.972–1.025)	0.8717
Gender: Male/Female	0.911	(0.354–2.349)	0.8476
Smoking: non-smoker/smoker	2.040	(0.739–5.632)	0.1687
Pathological stage	1.661	(1.225–2.253)	0.0011
VEGF-C status	1.801	(1.024–3.167)	0.0410
VEGF-A status	2.006	(1.144–3.519)	0.0152

Abbreviations: CI, confidence interval; LVD, lymphatic vessel density; MVD, microvessel density.

4. Discussion

During tumour progression, lymphangiogenesis and angiogenesis are considered to affect tumour biology, including metastasis.[4,5] Therefore, a comprehensive clinical study on lymphangiogenesis and angiogenesis was conducted in NSCLCs. The present study focused specifically on the clinical significance of lymphangiogenesis.

The monoclonal antibody D2-40 is a selective marker for lymphatic endothelium.[8,9] D2-40 antibody detects a fixation-resistant epitope on a 40-kDa O-linked sialoglycoprotein expressed in lymphatic endothelium, but not blood vessels.[9] For example, although LVI is very important in the stage preceding lymph node metastases, histological detection of lymph or blood vessel invasion using only HE-stained slides is very difficult. The staining of D2-40 is clear and useful for the detection of lymphatic vessels, as also shown in the present study. Therefore, immunohistochemical staining is clinically useful for evaluating the precise LVI. In addition, previous studies found that the centre of tumours does not contain functional lymphatics while the lymphatic vessels at the tumour margin do facilitate lymphatic spread of tumour cells.[5] The high lymphatic microvessel density (LVD) at the margins of the tumour is thought to indicate the probability of lymphatic metastasis.[5] However, there have only been a few clinical reports on lymphangiogenesis in NSCLCs.[10–12] Therefore, the present study evaluated lymphangiogenesis in NSCLCs, including morphological evaluations, such as lymphatic endothelial cells without lumen (Micro-LVD) or lymphatic vessels with lumen (Tubal-LVD).

Consequently, the present study revealed lymphangiogenesis, both Micro-LVD and Total-LVD, to be associated with tumour status, as reported previously.[11] In addition, the Micro-LVD and the Total-LVD are correlated with tumour differentiation in the present study. Then, all variables regarding lymphangiogenesis, such as Micro-LVD, Tubal-LVD and Total-LVD, are significantly associated with LVI. Furthermore, the Micro-LVD and the LVI were significantly associated with lymph node metastases. Although LVI could not be detected in all the cases with lymph node metastases, this result might be partly because complete serial slides of tumours could not be examined for analysis. Furthermore, regarding the lymphangiogenesis in relation to patient survivals, both the Micro-LVD and the LVI were significant prognostic factors for NSCLC patients. In contrast, neither Tubal-LVD nor Total-LVD was associated with the survival of NSCLC patients in the present study.

Amongst the various biomarkers associated with tumorigenesis, previous studies have demonstrated that the vascular endothelial growth factor (VEGF) family plays roles in the physiological and pathological regulation of angiogenesis and lymphangiogenesis.[13] VEGF-C, a member of VEGF family, is reported to mediate lymphangiogenesis.[14,15] Intriguingly, the present study demonstrated that the intratumoural VEGF-C expression was significantly associated with Micro-LVD, not with Tubal-LVD. In total, the intratumoural VEGF-C expression can initially induce lymphatic endothelial cells without lumen, resulting in lymph node metastases via the formation of lymphatic vessels with lumen. In fact, many clinical studies have revealed that the intratumoural VEGF-C expression is associated with lymph node metastases and a poor prognosis in cancer patients.[16–19]

The present study indicated that Micro-LVD is a useful indicator for tumour-induced lymphangiogenesis. In contrast, the Tubal-LVD was associated neither with the intratumoural VEGF-C expression nor with the patient survival. These results might be partly due to the fact that it is not easy

to distinguish tumour-induced lymphatic vessels from pre-existing lymphatic vessels. The present study is thus apparently the clinical report to demonstrate the clinical significance of lymphatic endothelial cells without a lumen in human cancers.

In the present study, 36.6% (30–82) of VEGF-C-negative tumours were Micro-LVD-high, VEGF-D, another member of VEGF family, might induce lymphangiogenesis amongst these VEGF-C-negative tumours.[20,21] However, the clinical significance of the intratumoural VEGF-D expression is still controversial.[19,22] Further studies should be performed to clarify the clinical significance of the VEGF-D expression in human cancers.

On the other hand, it is widely accepted that angiogenesis is essential for both tumour growth and metastasis.[4,23] In the absence of local angiogenesis, tumours cannot grow beyond 2 mm.[3,4] In addition, tumour angiogenesis has been reported to be associated with metastasis of primary tumours.[23] In fact, the present comprehensive study has demonstrated that both lymphangiogenesis and angiogenesis are independent prognostic factors for NSCLC patients. Therefore, it is considered to be clinically important to perform simultaneous evaluations for lymphangiogenesis and angiogenesis.[24]

With respect to the regulation of lymphangiogenesis and angiogenesis, experimental studies revealed that VEGF-A affects tumour angiogenesis.[25,26] In addition, many clinical studies, including the present study, have demonstrated that the intratumoural VEGF-A expression is associated with angiogenesis.[7,27] On the other hand, the intratumoural VEGF-C expression has been reported to correlate with lymphangiogenesis in human cancers, as observed in the present study.[11,28] The present study revealed that the expressions of VEGF-A and VEGF-C were not correlated with each other in NSCLCs. As a result, both the VEGF-A expression and the VEGF-C expression were independent prognostic factors for NSCLCs. During tumour progression, many tumour-associated biomarkers, such as mutations of K-ras and Wnt overexpression, can affect the overexpression of VEGF-A,[29] thus resulting in the induction of angiogenesis or lymphangiogenesis and thereby producing more aggressive tumours.

Recently, many molecular-target agents have been developed for the treatment of cancer patients.[3] Bevacizumab is a recombinant humanised version of the murine antihuman VEGF-A monoclonal antibody.[30] A randomised trial in NSCLC patients revealed that bevacizumab in combination with carboplatin plus paclitaxel improved the clinical outcome of patients with advanced or recurrent NSCLCs.[31] Considering its molecular biology, bevacizumab might be effective for VEGF-A-overexpressing tumours, which accounted for 46.9% of NSCLCs in the present study. On the other hand, 22.4% of carcinomas (33 of 147) were VEGF-A-negative and VEGF-C-positive. Therefore, new molecular-target agents against VEGF-C should be developed to control these VEGF-C-positive tumours in the near future.

Conflict of interest statement

None declared.

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