Original Article

Vascular Endothelial Growth Factor C as a Predictor of Early Recurrence and Poor Prognosis of Resected Stage I Non-small Cell Lung Cancer

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Abstract

Introduction: Stage I non-small cell lung cancer (NSCLC) is potentially curable after completely resection, but early recurrence may influence prognosis. This study hypothesises that vascular endothelial growth factor C (VEGF-C) plays a key role in predicting early recurrence and poor survival of patients with stage I NSCLC. Materials and Methods: The expression of VEGF-C was immuno-histochemically (IHC) analysed in tumour samples of primary stage I NSCLC and correlated to early recurrence (< 36 months), disease-free survival, and overall survival in all 49 patients. Results: Early recurrence was identified in 16 patients (33%), and the early recurrence rate in strong and weak VEGF-C activity was significantly different (*P* = 0.016). VEGF-C was also an independent risk factor in predicting early recurrence (HR = 3.98, *P* = 0.02). Patients with strong VEGF-C staining also had poor 3-year disease-free survival (*P* = 0.008) and overall survival (*P* = 0.007). Conclusion: Strong VEGF-C IHC staining could be a biomarker for predicting early recurrence and poor prognosis of resected stage I NSCLC, if the results of the present study are confirmed in a larger study. A more aggressive adjuvant therapy should be used in this group of patients.

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Key words: Early recurrence, Stage I NSCLC, Survival, VEGF-C

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide, with a 5-year survival of around 15%.[1] Approximately 20% of patients present with stage I disease (T1N0M0 or T2N0M0) and undergo potentially curative surgical resection.[2] However, approximately 30% to 40% of patients with stage I NSCLC after completely resection relapse and ultimately die of recurrent disease.[3,4] Early postoperative recurrence, often defined as relapse within 36 months after surgery, occur with high variety in resected stage I disease.[5] Although adjuvant chemotherapy clearly improves disease-free survival in Stage IB patients, an absolute overall survival benefit has not yet been conclusively established.[6,7]

In addition, a percentage of Stage IA patients develop recurrent disease within a short period but are not routinely administered adjuvant therapy according to recent treatment guidelines.[3] Clinical or biological markers that are predictive of early recurrence in these patients must be identified. If such markers are available, stage I patients at high-risk of recurrence can be selected to receive adjuvant chemotherapy while those at very low-risk are spared the treatment toxicity.

Vascular endothelial growth factor C (VEGF-C), a member of the VEGF family, is believed to be the main specific lymphangiogenic factor, which activates both

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vascular endothelial growth factor receptor 2 (VEGFR-2) and VEGFR-3. VEGF-C-induced lymphangiogenesis mediates tumour cell dissemination and the formation of lymph node metastases.[8] Loco-regional or distant recurrences of NSCLC are strongly associated with tumour micro-metastasis,[9] which cannot be detected by clinical methods applied in daily practice. Many studies have revealed that VEGF-C over-expression is related to poor prognosis of NSCLC,[10,11] but to our knowledge, only a limited amount of research has focused on the role of VEGF-C in predicting early recurrence and survival, or the use of adjuvant chemotherapy for stage I NSCLC patients who received surgical resection.

This study aimed to investigate the relationship between VEGF-C expression and postoperative early recurrence and survival of patients with stage I NSCLC.

Materials and Methods

Tissue Samples and Patients

The institutional review board of China Medical University Hospital approved the study and waived the need for individual patient consent. Patients who underwent incomplete or no resection were excluded to eliminate the potential influence of residual disease. From January 1997 to December 2007, 49 patients with pathologic stage IA and IB primary NSCLC underwent complete resection without any preoperative therapy at the China Medical University Hospital and the National Taiwan University Hospital. There were 36 patients before December 2006 and 13 patients after January 2007. Because there were limited numbers of well-stored specimens from patients with NSCLC who received operation with well-stored specimens in China Medical University Hospital before 2007, 23 specimens were obtained from a pathologist in National Taiwan University Hospital. From January 1997 to December 1999, there were about 200 lung cancer patients who underwent surgical resection in National Taiwan University Hospital. The pathologist who co-operated with us collected the 23 patients based on his clinical follow-up consecutively without selective bias. Specimens from China Medical University Hospital were identified based on consecutive patients from the tissue bank registry. All the available specimens were included for analysis and were also without selective bias. The operations were done by the 2 chief surgeons individually in the 2 hospitals, and they all followed the same principle of radical lobectomy including radical lymph nodes dissection. Of the 49 patients included, there were 30 males and 19 females, with ages ranging from 42 to 82 years (average age, 64 years). Systematic lymph node dissection was done according to Naruke's lymph node mapping. We performed radical lobectomy including radical lymph nodes dissection. In every patient, an average of 15 lymph nodes was sampled from at least 4 stations. Over the past 10 years, mini-thoracotomy was the main surgical procedure before 2005. In recent 5 years, 80% of lung cancer patients received video-assisted thoracoscopic surgery (VATS), but the principle of lymph nodes sampled stations and dissection was all the same. Pathologic staging was based on the 2002 UICC tumour, node, and metastatic (TNM) classification. Cancer tissue samples from the 49 patients and their clinico-pathologic features are summarised in Table 1.

Immuno-histochemical Staining

The primary antibody used in this study was the rabbit anti-human polyclonal VEGF-C antibody at 1:100 dilution (Invitrogen, USA). Formalin-fixed and paraffin-embedded sections of the tissue samples were deparaffinised, followed by immuno-histochemical staining using the streptavidin-biotin-peroxidase complex method. Negative controls were done by replacing the primary antibody with phosphate buffered solution (PBS).

Table 1. Patient Characteristics

	N	%
Patients	49	
Age		
Mean		
≤ 60 year	17	35
>60 year	32	65
Gender		
Men	30	61
Women	19	39
Tumour size		
≤ 3 cm	19	39
>3 cm	30	61
Histological subtype		
Adenocarcinoma	34	69
SqCC	12	25
BAC	3	6
Recurrence		
≤ 36 months	16	33
>36 months	33	67
Recurrence site		
Locoregional	11	22
Distant	11	22
No predicted recurrence	27	56

Two independent pathologists blinded to the clinical data examined all sections. The evaluation of VEGF-C immunoreactivity was done according to previous publication of Ito et al.[12] The degree of VEGF-C immunoreactivity was considered to be positive if unequivocal staining of the membrane or cytoplasm was seen in more than 10% of the tumor cells. The percentage of positive cells was recorded in a number of fields at 200× magnification. VEGF-C immunostaining was graded as negative (0), mild (1+), moderate (2+), and severe (3+) according to the degree of staining. Negative and mild staining intensities were further defined as weak VEGF-C staining, while others were defined as strong VEGF-C staining.[13] There was a high concordance rate (93.8%) for all the samples. If there were different results, we consulted a third pathologist and made the final decision together.

Follow-up Examinations

All patients were completely followed-up for least 3 years at 1-to 3-month intervals without default. During each follow-up visit, the patient underwent clinical evaluation, blood cell count and biochemistry examination if indicated, chest radiography or computed tomography (CT), whole-body bone scan, and PET-CT if necessary. Detection of suspected recurrence at any one site was followed by a thorough detailed investigation to confirm or rule out the diagnosis.

Definition of Loco-regional and Distant Recurrence

Recurrent patterns were classified into 2 categories: loco-regional and distant recurrence. Loco-regional recurrence included cancer development in the bronchial stump and hilar, mediastinal, and supraclavicular lymph node areas. Distant recurrence categorised metastasis to lung, brain, bone, liver, adrenal, pleural, and other organs.[1]

The first recurrence noted within 36 months was defined as early recurrence. In this study, if the recurrence site was on a different lobe of lung and had the same histology, it was classified as late distant recurrence even though it may have been a metachronous tumor according to Martini's criteria.[14]

Endpoint and Statistical Analysis

Overall survival was measured from the date of surgery to the date of death or the most recent follow-up. The disease-free interval was measured from the date of surgery to the date of recurrence or the date of death. The early recurrence was measured from the date of surgery to the date of early recurrence.

Survival curves were estimated by using the Kaplan-Meier method and the statistical differences were analysed using the log-rank test. Overall survival and the disease-free survival were estimated at 3-year follow-up; early recurrence rate was estimated 3-year early recurrence rate (1-survival rate). Cox regression analysis was performed to determine the risk factors of postoperative early recurrence of stage I lung cancer. Analyses were performed using SPSS 13.0 version. A value of $P < 0.05$ was considered statistically significant for all procedures.

Results

Patient Characteristics

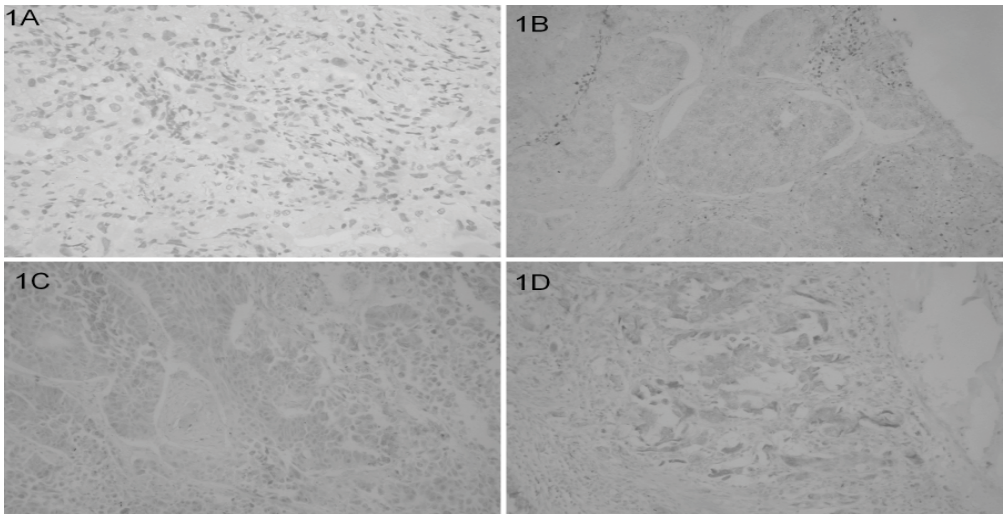
The clinical and pathologic characteristics of the patients are summarised in Table 1. Of the 49 patients with stage I disease, 30 were males and 19 were females, with a mean age of 64 years (range, 42 to 82). Early recurrence was identified in 16 patients (33%) during the 3-year follow-up. There were 34 (69%) adenocarcinoma, 12 (25%) squamous cell carcinoma (SqCC), and 3 (6%) bronchiolo-alveolar cell carcinoma (BAC). Tumours of size <3 cm were diagnosed before resection in 19 patients (39%).

Results of Immuno-histochemistry

VEGF-C protein was observed mainly in the cytoplasm of lung cancer cells (Figs. 1A to 1D) but there was no staining in normal lung tissues. Of the 49 patients, 24 (49%) had strong VEGF-C staining.

Early Recurrence

Early recurrence was identified in 16 patients (33%) during the 3-year follow-up. Eleven patients suffered from loco-regional recurrence while another 11 had distant metastasis to the pleura, bone, brain, breast, esophagus, and epididymis. We quartered all the specimens into negative (0), mild (1+), moderate (2+) and severe (3+), the 3-year recurrence rate between them achieved a trend of difference (3/17, 18% vs 2/9, 22% vs 3/10, 30% vs 8/13, 62%, respectively) ($P = 0.067$). When we further used the dichotomy (negative and mild as weak VEGF-C staining vs moderate and severe as strong VEGF-C staining), the 3-year recurrence rate in strong and weak VEGF-C activity was significantly different. (12/24, 50% vs 4/25, 16%, respectively) ($P = 0.016$). Furthermore, in the early recurrence group, the rate of patients with strong VEGF-C staining was higher than that of patients with weak staining (12/16, 75% vs 4/16, 25%, respectively). Using COX regression analysis, strong VEGF-C staining was an independent factor in predicting early recurrence in resected stage I NSCLC by either univariate (HR = 3.66, $P = 0.02$) or multivariate (HR = 3.98, $P = 0.02$) analysis (Table 2). There was significant



Figs. 1A to 1D. IHC staining of VEGF-C grade from negative (1A), mild (1B), moderate. (1C) and severe (1D) in the cytoplasm of lung adenocarcinoma.

Table 2. Cox Regression Analysis for Early Recurrence

Risk factor	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gender (female/male)	1.27 (0.47-3.40)	0.6396	1.21 (0.40-3.63)	0.7396
Age	1.00 (0.95-1.05)	0.8479	1.00 (0.95-1.06)	0.9142
Stage (Ib/Ia)	1.99 (0.64-6.16)	0.2346	2.20 (0.65-7.49)	0.2074
Pathology (Adeno and BAC/Squamous)	0.73 (0.25-2.11)	0.5662	1.32 (0.36-4.76)	0.6739
VEGF-C strong (+/-)	3.66 (1.18-11.39)	0.0248	3.98 (1.23-12.89)	0.0212

difference in recurrence curves among patients with different VEGF-C expressions ($P = 0.006$) (Fig. 2).

Disease-free and Overall Survival

The 3-year disease-free survival and overall survival rates of all patients were 65% and 59% respectively. The 3-year disease free survival rates in patients with VEGF-C IHC staining strong and weak activity were 46% and 84%, respectively, achieving a statistically significant difference ($P = 0.008$) (Fig. 3). The 3-year overall survival rates between the 2 groups were 38% and 80%, respectively, and also achieving a statistically significant difference ($P = 0.007$) (Fig. 4).

Discussion

Although stage I disease currently accounts for only 20% of newly diagnosed NSCLC, this group has the best postoperative survival and its proportion is likely to increase if computed tomography (CT) is established as a screening modality for lung cancer in the high-risk groups in the near future. Prognostic factors, including clinical and biologic markers, allow risk stratification within stage I to improve the design of prospective adjuvant studies and ultimately be used in clinical practice. Goodgame et al[3] reported that tumour size >3 cm, surgery other than lobectomy, non-squamous histology, and high grade of malignancy are clinical factors. They also reported the predictive value of VEGF-C in the early recurrence of resectable N2 NSCLC.[1]

To the best of our knowledge, there remains limited published research which is focused on the importance of VEGF-C, including predicting early recurrence, disease-free survival, or overall survival in stage I lung cancer. Gu et al[15] reported that positive IHC staining of cytokeratin and P53 in lymph node sampling from pathologic stage IA and IB NSCLC patients reflect the underestimated micro-metastasis to regional lymph nodes and indicate early recurrence and poor overall survival. Noda et al[16] showed that VEGF-C expression is an independent risk factor for local recurrence of rectal carcinoma and that patients with VEGF-C positive tumours have significantly worse prognosis than those with VEGF-C negative tumours. Arinaga et al[17] also found that high VEGF-C and VEGFR-3 expression may be indicative of poor survival rates for patients with NSCLC, but the VEGF-C expression was greater (76.1%) in their patients than that reported previously

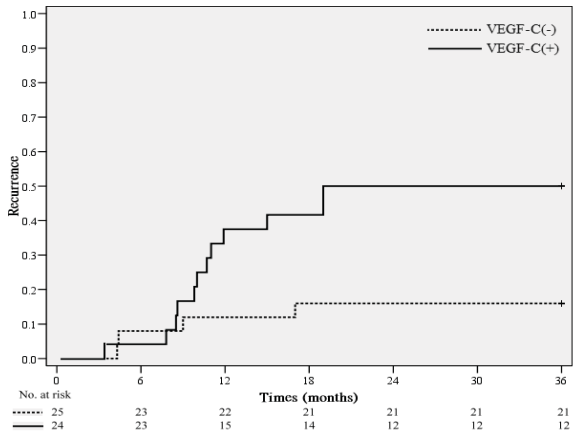


Fig. 2. Recurrence curve in patients with different VEGF-C expression ($P = 0.006$).
(VEGF-C (+) refer to strong staining and VEGF-C (-) refer to weak staining).

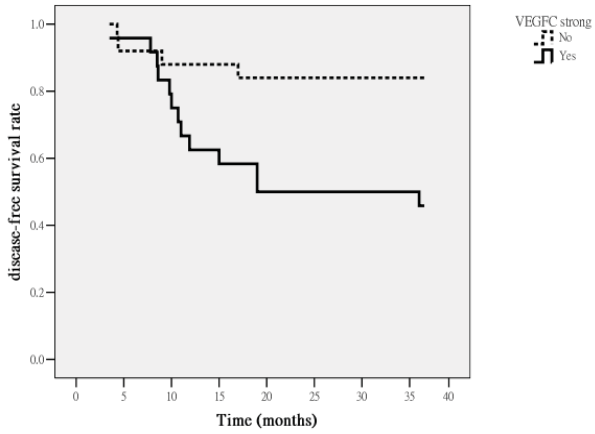


Fig. 3. Three-year Disease-free survival in patients with different VEGF-C expression. ($P = 0.008$).

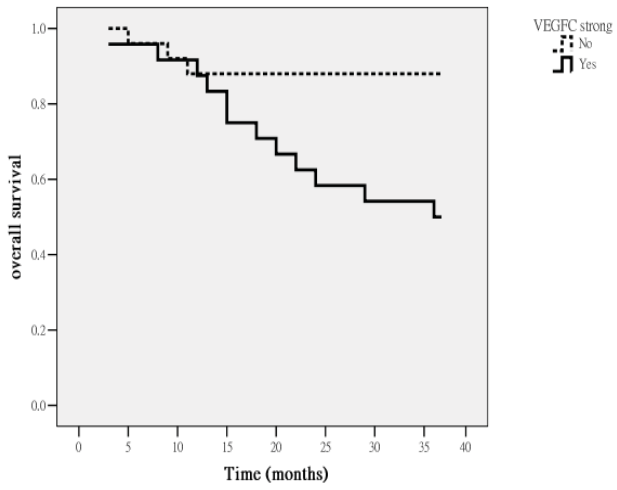


Fig. 4. Three-year Overall survival in patients with different VEGF-C expression. ($P = 0.007$).

by Kajita et al[18] (38.7%) and Ohta et al[19] (45.1%). The frequency in their cohort included large numbers of patients with adenocarcinoma, which exhibits significantly greater VEGF-C expression rate when compared with other tumor tissue types. In our study, we included 34 adenocarcinoma (69%), and the VEGF-C strong expression rate was 49%. The rate of adenocarcinoma was less than Arinaga et al,[13] and this may account for the difference in results. The primary antibody used by Arinaga et al (rabbit polyclonal antibody; Immuno-Biological Laboratories Company Ltd, Gunma, Japan) was different from other studies, which also may explain the unexpected results. The authors also used multivariate analysis indicated to prove that VEGF-C expression tends to be a poor prognostic factor. Thus, our study hypothesises that VEGF-C also plays an important role in the early recurrence and survival of completely resected stage I NSCLC.

In this study, 49 patients with stage I NSCLC were retrospectively analysed. All underwent complete resection, and IHC staining was done on the specimens. The 3-year disease free survival and overall survival of all patients was 65 % and 59 %, respectively, which was similar to another study.[7] Adenocarcinoma was the predominant cell type up to 69% of the time in our study and also compatible with the epidemiology worldwide in early stage of lung cancer.[20] Results showed that high VEGF-C staining was not only an independent biomarker to predict early tumour recurrence within 36 months, but it also warned of poor 3-year disease-free survival and overall survival.

The limitations of this study include that we utilised a retrospective cohort and small number of patients. Due to the study time frame (1997 to 2007), some patients did not receive PET scan for preoperative evaluation or postoperative follow-up. This variable may have caused an underestimation of the preoperative staging or postoperative recurrence. There were totally 112 patients with pathologic stage IA and IB primary NSCLC underwent surgical resection, but due to the tissue bank of our hospital not being well established before 2007, many samples were not preserved. Finally, there were only 49 patients were enrolled. There are other possible prognostic factors such as smoking status, lymphovascular invasion, grade of differentiation, proximity to visceral pleura that may influence the prognosis. But due to the design of our research, these data are not available in this article. Long-term survival in patients with stage I NSCLC is also affected by a relatively high rate of diagnosis of second primary lung cancers.[21] Although distinguishing between second primary lung cancer and primary disease recurrence is difficult and has no general consensus, censoring these events as much as possible allows for an optimal definition of risk factors for disease recurrence. More large-scale clinical studies are warranted

to confirm the results here.

In summary, high VEGF-C expression by IHC staining is an independent factor that may predict the early recurrence of completely resected stage I NSCLC within 36 months, and a relatively poor 3-year disease-free and overall survival. More aggressive adjuvant chemotherapy or radiotherapy should be considered for this group in clinical practice of patients to improve disease control and survival.

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