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Prognostic Impact of Vascular Endothelial Growth Factor-A and E-Cadherin Expression in Completely Resected Pathologic Stage I Non-Small Cell Lung Cancer

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Objective: The purpose of this study was to evaluate the value of vascular endothelial growth factor-A and E-cadherin expression as well as other confirmed prognostic factors in predicting the clinical outcome after definitive surgery of pathologic stage I non-small cell lung cancer.

Methods: One hundred and eighty-five consecutive and non-selected patients who underwent definitive surgery for stage I non-small cell lung cancer in our institute were included in this study. Formalin-fixed paraffin-embedded specimens were stained for vascular endothelial growth factor-A and E-cadherin and the correlation between the staining, its clinicopathological parameters and its prognostic power were analyzed statistically.

Results: Of the 185 patients studied, 92 cases (49.7%) were strongly positive for vascular endothelial growth factor-A. Vascular endothelial growth factor-A expression was only related to visceral pleural involvement ($P < 0.001$). A total of 95 carcinomas (51.4%) were E-cadherin-negative tumors. E-cadherin expression correlated with histology ($P < 0.001$), tumor size ($P = 0.001$) and visceral pleural involvement ($P < 0.001$). In univariate analysis by log-rank test, gender, tumor size, lymphovascular invasion, visceral pleural involvement, vascular endothelial growth factor-A expression and E-cadherin expression were significant prognostic factors ($P = 0.003$, 0.042, 0.026, 0.035, 0.008 and 0.006, respectively). In multivariate analysis, gender, vascular endothelial growth factor-A and E-cadherin expression maintained its independent prognostic influence on overall survival ($P = 0.013$, <0.001 and 0.036, respectively).

Conclusions: Expression of vascular endothelial growth factor-A is related to visceral pleural involvement, and E-cadherin expression correlates with histology, tumor size and visceral pleural involvement. Multivariate analysis confirmed gender, vascular endothelial growth factor-A and E-cadherin expression were significant predictive factors for overall survival in completely resected pathologic stage I non-small cell lung cancer.

Key words: non-small cell lung cancer – vascular endothelial growth factor-A – E-cadherin

INTRODUCTION

The incidence of lung cancer in 2007 is estimated to be 213 380 with 160 390 deaths in the USA. It will contribute to 31% of male and 26% of female cancer-related deaths and is the largest cause of cancer-related mortality in both men and women (1). Non-small cell lung cancer (NSCLC) accounts for ~75% of all cases of lung cancer, which is one of the most common tumors affecting humans in the world.

Surgical resection still remains the most effective therapy for patients with NSCLC limited to the lung, especially stage I disease. However, reported survival outcome for resected stage I NSCLC may not be enough to be satisfied. According to several large studies, 5-year survivals in resected stage IA and IB disease were 67–89% and 57–75%, respectively (2–7). There is some variation in the survival data in the literature. One of the reasons for this variation is that stage I NSCLC includes populations with a different grade of malignancy (8). This observation underlines how important it is to identify novel pathological parameters in addition to disease stage and, most of all, new biological markers, in order to add further prognostic information, select high-risk patients for aggressive adjuvant treatments and set new anticancer therapies (9).

Owing to developments in molecular biology, many clinical studies on molecular markers associated with tumor biological behavior have been performed in human cancers, and these molecular markers, including oncogenes, tumor suppressor genes (10), metastatic suppressor genes (11) and angiogenetic factors (12,13), could be prognostic factors for NSCLC patients. Various angiogenic mechanisms may be differentially important in different tumor types and/or stages of neoplastic progression (14). However, one of the major pathways involved in angiogenesis is the vascular endothelial growth factor (VEGF and VEGFR) family of proteins and receptors (15). 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 (16). E-cadherin is one of the metastatic suppressor genes (11,17). In experimental studies, highly invasive tumor cells have been shown to lose their invasiveness when transfected with a normal E-cadherin gene, localized on chromosome 16q22.1 (18). In one study by Böhm et al. (19), reduced expression of E-cadherin was found to be associated with moderately and poorly differentiated squamous and small cell carcinoma in a limited number of patients with lung cancer. The aim of this study was to evaluate the value of VEGF-A and E-cadherin expression as well as other confirmed prognostic factors in predicting the clinical outcome after definitive surgery of pathologic stage I NSCLC in Chinese population.

PATIENTS AND METHODS

Patients and Tissues

Between April 2003 and December 2005, a total of 185 consecutive and non-selected patients who underwent definitive surgery for NSCLC at the Shanghai Chest Hospital affiliated to Shanghai Jiaotong University with a confirmed pathologic stage of Stage I was reviewed retrospectively. All patients had pathologically confirmed squamous cell carcinoma, adenocarcinoma, adenosquamous cell carcinoma, bronchioloalveolar carcinoma (BAC) or large cell carcinoma. Small cell lung cancer and cell types of undetermined histology were excluded from this analysis.

Preoperative evaluation and staging work-ups included a complete history and physical examination, bronchoscopy, complete blood counts, serum biochemistry tests, computed tomography of thorax, ultrasound of upper abdomen, brain magnetic resonance imaging, total body bone scan or fluorodeoxyglucose-positron emission tomography scan. The tumors were resected with no pathologic evidence of residual tumor at the surgical margin. The majority of patients underwent lobectomy or pneumonectomy for resection of the primary lesion. In cases of lesser pulmonary resection (wedge resection or segmentectomy), segmental or subsegmental nodes were pathologically evaluated by intraoperative frozen-section examination and confirmed as negative.

The extension of pulmonary resection and mediastinal lymph node removal were decided at the time of operation by operating surgeons and the general physical condition of the patients. Systematic mediastinal lymphadenectomy is described by Martini (20). In patients received mediastinal lymph node sampling, the resection was combined with a regional lymph node dissection of interlobular, peribronchial and hilar nodes representing nodes 10, 11 and 12, respectively, according to the lymph node mapping proposed by Naruke et al. (21). A mediastinotomy was performed through longitudinal incision of the mediastinal pleura, and nodes of regions two to nine were explored. Any nodes showing evidence of cancer were removed and submitted for pathohistologic analysis. For right-sided tumors, nodes of regions three, four and seven, and for left-sided tumors, nodes of regions five, six and seven were removed routinely in all patients.

All excised specimens were formalin-fixed and sliced at 10 mm intervals. Pathologic staging was determined according to the International Union Against Cancer TNM classification of malignant tumors (22). The degrees of visceral pleural involvement was defined according to Hammar's staging classification, as follows: p0, a tumor that does not penetrate the elastic layer of the visceral pleura; p1, a tumor that penetrates the elastic layer but is not exposed on the pleural surface; and p2, a tumor that is exposed on the pleural surface but does not involve the parietal pleura (23). As the role of adjuvant chemotherapy for NSCLC was controversial at the time of the treatment of this group of patients, the utilization of chemotherapy was not standardized. The adjuvant chemotherapy was decided by doctors and the general physical condition of the patients. Adjuvant radiation therapy was not used in this group of patients.

Immunohistochemistry

The following antibodies were used, along with isotype antibodies as negative controls: a rabbit polyclonal antibody for

VEGF-A (A-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:200, and a mouse monoclonal antibody for E-cadherin (HECD-1, Takara, Otsu, Japan) diluted at 1:400. Formalin-fixed paraffin-embedded tissue was cut into 4 mm sections and mounted onto poly-L-lysine-coated slides. Sections were deparaffinized 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 for 20 min. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 min, the sections were treated for 2 h at room temperature with 5% bovine serum albumin to block non-specific staining. Duplicate sections were incubated overnight with the primary specific antibodies detecting VEGF-A and E-cadherin. Slides were then incubated for 1 h with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) for E-cadherin and biotinylated anti-rabbit IgG (Vector Laboratories Inc.) for VEGF-A.

The sections were incubated with the avidin–biotin–peroxidase complex (Vector Laboratories Inc.) for 1 h, and antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Lastly, the sections were lightly counterstained with Mayer's hematoxylin. Sections of resected lung tumors known to express VEGF-A or E-cadherin were used as positive controls for immunohistochemic staining, respectively.

Assessment of Immunohistochemical Staining

All of the immunostained sections were reviewed by two pathologists (G.-L.B. and H.-F.S.) who had no knowledge of the patients' clinical status. For VEGF-A and E-cadherin, samples were classified into two groups, positive or negative, with a cut-off value based on the findings of previous reports, respectively. At least 200 tumor cells were scored per ×40 field. All sections were scored in a semi-quantitative manner according to the method described previously, which reflects both the intensity and the percentage of cells staining at each intensity (24). Intensity was classified as 0 (no staining), +1 (weak staining), +2 (distinct staining) or +3 (very strong staining). A value designated the 'HSCORE' was obtained for each slide by using the following algorithm: HSCORE = $\Sigma(I \times \mathrm{PC})$, where I and PC represent the staining intensity and the percentage of cells that stain at each intensity, respectively, and the corresponding HSCOREs were calculated separately. Expression of VEGF-A and E-cadherin was classified as follows: when ≥30% of the carcinoma cells in a given specimen were positively stained for VEGF-A, the sample was classified as VEGF-A-positive (13); and when ≥50% of the carcinoma cells in a given specimen were positively stained for E-cadherin, the sample was classified as E-cadherin-positive (25).

Statistical Analysis

Correlations between clinicopathological factors and VEGF-A or E-cadherin expression were analyzed using Fisher's exact probability test or χ^2 test. Survival was calculated by the Kaplan–Meier method, and differences in survival were determined by the log-rank analysis. A multivariable analysis of several independent prognostic factors was carried out using Cox's proportional hazards regression model (26). Zero time was the date of pulmonary resection,

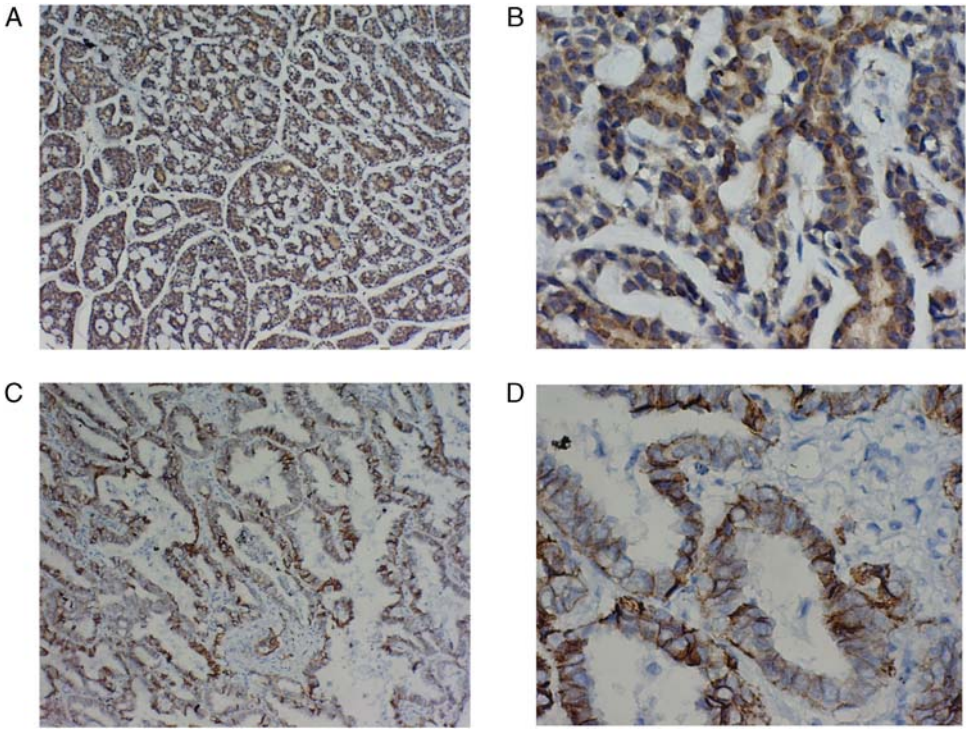

Figure 1. Strong vascular endothelial growth factor-A (VEGF-A) immunostaining in moderate differentiated, acinar adenocarcinoma (A, ×100; B, ×400) and E-cadherin immunostaining in well-differentiated, mixed adenocarcinoma (C, ×100; D, ×400).

and the terminal event was death attributable to cancer. Significance was defined as $P < 0.05$. The statistical data were obtained using an SPSS software package (SPSS 11.5 Inc., Chicago, IL, USA).

RESULTS

Relation of Clinicopathologic Factors According to VEGF-A and E-cadherin Expression

A total of 185 patients with completely resected pathologic stage I NSCLC were included in this study. There were 53 patients with squamous cell carcinoma of the lung, 12 cases with hilar and 41 with peripheral types. The median age was 58 (range, 32–80) years, with 115 males and 70 females. Median follow-up time was 51 months (range, 32–64 months). Of the 185 patients studied, 92 cases (49.7%) were strongly positive for VEGF-A (Fig. 1A and B). Sixteen cases (8.6%) were negative and 77 cases (41.6%) revealed unclear weak reactions, the 93 cases were included in the negative group (50.3%). There were 55.3% (63 of 114) of cases with adenocarcinoma, 50.0% (5 of 10) of cases with adenosquamous cell carcinoma, 33.3% (1 of 3) of cases with BAC and 40.0% (2 of 5) of cases with large cell carcinoma strongly positive for VEGF-A. There was no difference in VEGF-A status in relation to age, gender, smoking status, histology, tumor size or lobe location. However, the frequency of visceral pleural involvement was significantly higher in VEGF-A-positive tumors than in VEGF-A-negative tumors (87.0% vs. 59.1%, $P < 0.001$).

E-cadherin expression was predominantly confined to the cell membrane in some of the tumors, whereas in others, E-cadherin expression was diffusely cytoplasmic. In the vast majority, however, the staining pattern was membranous as well as cytoplasmic (Fig. 1C and D). A total of 95 carcinomas (51.4%) were E-cadherin-negative tumors. There were 57.9% (66 of 114) of cases with adenocarcinoma, 60.0% (6 of 10) of cases with adenosquamous cell carcinoma, 33.3% (1 of 3) of cases with BAC and 40.0% (2 of 5) of cases with large cell carcinoma strongly positive for E-cadherin. E-cadherin expression correlated with histology ($P < 0.001$), tumor size ($P = 0.001$) and visceral pleural involvement ($P < 0.001$). No other clinicopathological parameter was related to E-cadherin expression. The relationship between clinicopathological features and VEGF-A or E-cadherin expression is shown in Table 1.

Table 1. Immunohistochemical status of VEGF-A and E-cadherin in patients with Stage I NSCLC ($n = 185$)

Characteristics	No. of patients	VEGF-A expression			E-cadherin expression		
		Positive	Negative	*P* value	Positive	Negative	*P* value
Age							
≤65	110	50	60	0.159	51	59	0.451
>65	75	42	33		39	36	
Gender							
Female	70	40	30	0.116	35	35	0.774
Male	115	52	63		55	60	
Smoking status							
Nonsmoker	92	48	44	0.508	45	47	0.943
Smoker	93	44	49		45	48	
Histology							
Squamous	53	21	32	0.081	15	38	<0.001
Non-squamous	132	71	61		75	57	
Tumor size (T)							
T1	27	14	13	0.881	21	6	0.001
T2	158	78	80		69	89	
Lobe location							
Right lobe	106	55	51	0.497	50	56	0.641
Left lobe	79	37	42		40	39	
Visceral pleural involvement							
Yes	135	80	55	<0.001	46	89	<0.001
No	50	12	38		44	6	

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

Table 2. Univariate analysis of factors associated with overall survival in patients with NSCLC

Characteristics	*P* log-rank test
Age	
≤65 vs. >65	0.548
Gender	
Female vs. male	0.003
Smoking status	
Nonsmoker vs. smoker	0.438
Histology	
Squamous vs. non-squamous	0.062
Tumor size (T)	
T1 vs. T2	0.042
Type of resection	
LP vs. WRS	0.648
Lobe location	
Right lobe vs. left lobe	0.303
Lymph node removal	
SML vs. MLS	0.095
Lymphovascular invasion	
Yes vs. no	0.026
Adjuvant chemotherapy	
Yes vs. no	0.147
Visceral pleural involvement	
Yes vs. no	0.035
VEGF-A	
Positive vs. negative	0.008
E-cadherin	
Positive vs. negative	0.006

LP, Lobectomy or pneumonectomy; WRS, wedge resection or segmentectomy; SML, systematic mediastinal lymphadenectomy; MLS, mediastinal lymph node sampling.

Determination of Independent Factors Affecting Prognosis

In univariate analysis by log-rank test, gender, tumor size, lymphovascular invasion, visceral pleural involvement, VEGF-A expression and E-cadherin expression were significant prognostic factors ($P = 0.003$, 0.042, 0.026, 0.035, 0.008 and 0.006, respectively) (Table 2). A multivariate analysis was performed to evaluate the independent prognostic roles of VEGF-A and E-cadherin expression after adjusting for other significant covariates. All variables that significantly affected survival in univariate analysis were introduced into a Cox proportional hazard model (Table 3). At the end of the stepwise process, gender, VEGF-A expression and E-cadherin expression maintained its independent prognostic influence on overall survival ($P = 0.013$, <0.001 and 0.036, respectively).

The 5-year survival rate for patients with VEGF-A-negative expression ($n = 93$) was 85.7%, whereas patients with VEGF-A-positive expression ($n = 92$) was 54.8% ($P = 0.008$). Patients with E-cadherin-positive expression ($n = 90$) was associated with a better survival than those with E-cadherin-negative expression ($n = 95$; 81.2% vs. 54.7%, $P = 0.006$). Female patients ($n = 70$) were associated with an improved survival than men's ($n = 115$; 82.9% vs. 58.1%, $P = 0.003$) (Fig. 1).

DISCUSSION

Although the pathologic TNM staging system has been considered to have an impact on survival in NSCLC, problems such as overlapping prognosis for patients with different tumor stages and heterogeneous prognosis for patients with the same tumor stage have been pointed out (3,7). These problems need to be resolved to provide reasonable adjuvant treatment or follow-up care for each patient with differing risk of relapse or tumor death. Recent studies on molecular biology in human cancers have revealed that many molecules affect various biological behaviors of malignant

Table 3. Multivariate analysis of factors associated with overall survival in patients with NSCLC

Factors	Characteristics		Hazard ratio	95% CI	*P* value
	Unfavorable	Favorable			
Gender	Male	Female	1.52	1.02–2.25	0.013
Tumor size	T2	T1	1.56	0.88–2.76	0.228
Lymphovascular invasion	Yes	No	1.31	0.95–1.81	0.143
Visceral pleural involvement	Yes	No	0.81	0.40–1.63	0.549
VEGF-A	Positive	Negative	1.83	1.25–2.69	<0.001
E-cadherin	Negative	Positive	1.51	1.03–2.22	0.036

CI, confidence interval.

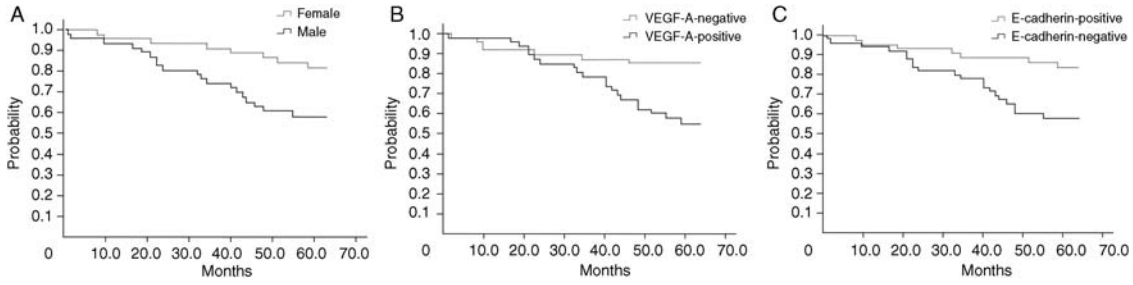

Figure 2. Cumulative Kaplan–Meier curves for overall survival, stratified according to gender (A, $P = 0.003$), VEGF-A expression (B, $P = 0.008$) and E-cadherin expression (C, $P = 0.006$).

tumors. For example, the activation of oncogenes or the inactivation of tumor suppressor genes, such as K-ras mutation and p53 mutation, could initially cause malignant progression (10). In addition, reduced expression of metastatic suppressor genes, such as E-cadherin (11,25), could induce tumor cells with high metastatic potential. In addition, tumor angiogenesis, affected by various angiogenetic factors such as the VEGF family (16), is associated with not only tumor growth but also tumor metastasis (27).

High VEGF-A expression and high angiogenic activity were noted in ~30–66% of NSCLC patients (28–30), and 49.7% of our series were defined as positive. In this study, we observed that VEGF-A expression using immunohistochemistry was significantly associated with visceral pleural involvement. A number of studies have demonstrated that VEGF-A expression is associated with angiogenesis and/or has prognostic significance in solid tumors including breast (31), colorectal (32), gastric (33,34) and pancreatic cancers (35). The results of the present work confirm previous findings of an important role for tumor cell VEGF-A expression in the angiogenic process of, and as a prognostic factor for, NSCLC (28,36–38). Anti-E-cadherin antibodies can induce invasive behavior in tumor cells and this supports the notion that impaired E-cadherin expression on tumor cells can be associated with malignant behavior, i.e. local invasiveness (39). Clinical studies in patients with a wide variety of human malignancies have shown that reduced E-cadherin expression is associated with dedifferentiation and lymphogenous spread of the tumor (11,40–43). The present data have shown that reduced expression of E-cadherin in early NSCLC is significantly correlated with poor survival ($P = 0.006$) (Fig. 2C), which confirms previous findings (29).

Previous studies that have addressed the effect of gender on the outcome of lung cancer have suggested that female gender carries some survival advantage. This study confirms that women had statistically better outcomes than that of men ($P = 0.003$) (Fig. 2A). Several studies have reported gender-specific differences in survival in surgically treated patients with NSCLC, with women uniformly having better outcomes (44–46). The reasons for this survival advantage have not been identified but most likely due to a variety of factors. It was reported that the only statistically significant relationship could be found between survival rate and female gender plus a lower level of lymph node spreads according to a Cox analysis post surgery (47). Exogenous or endogenous estrogens (48), gene and emotional factors may also play important roles in the development and survival of the lung cancer for women (49).

In conclusion, expression of VEGF-A is related to visceral pleural involvement, and E-cadherin expression correlates with histology, tumor size and visceral pleural involvement. Multivariate analysis confirmed gender, VEGF-A and E-cadherin expression were significant predictive factors for overall survival in completely resected pathologic stage I NSCLC.

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Conflict of interest statement

None declared.

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