RESEARCH ARTICLE

Prognostic Values of VEGF and Endostatin with Malignant Pleural Effusions in Patients with Lung Cancer

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Abstract

Aims: Angiogenesis is important in malignant pleural effusion (MPE) formation and it is regulated by a number of pro- and anti-angiogenic cytokines. The purpose of this study was to evaluate the prognostic value of angiogenic factor vascular endothelial growth factor (VEGF) and angiogenesis inhibitor endostatin in lung cancer patients with MPE, and investigate the relationship between these two kinds of agent. Methods: Using enzyme-linked immunoadsorbent assay, the concentrations of VEGF and endostatin were measured in pleural effusions (PE) and serum from a total of 70 lung cancer patients with MPE and 20 patients with tuberculosis. Results: Compared to patients with tuberculosis, the levels of VEGF and endostatin in both PE and serum were significantly higher in patients with lung cancer. There were statistically significant correlations between VEGF levels in PE and serum (r=0.696, $p<0.001$), endostatin levels in PE and serum (r=0.310, $p=0.022$), and VEGF and endostatin levels in PE (r=0.287, $p=0.019$). Cox multivariate analysis revealed that elevated pleural VEGF and endostatin levels and serum endostatin level were independent predictors of shorter overall survival. Conclusion: Both pro- and anti-angiogenic factors are likely contributors to PE formation. Our results suggest that the levels of VEGF and endostatin in PE, together with endostatin in serum, may be potential prognostic parameters for lung cancer patients with MPE.

Keywords: Malignant pleural effusion - lung cancer - prognosis - vascular endothelial growth factor - endostatin

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Introduction

Lung cancer is the leading cause of cancer-related death in the world. Approximately 15% of lung cancer patients have malignant pleural effusion (MPE) at the time of initial diagnosis and 50% develop MPE later in the course of their disease (Memon et al., 1981). Patients with MPE have a particularly poor prognosis. In the seventh edition of the TNM Classification for Lung Cancer, pleural involvement with effusion has been reclassified from T4, stage IIIB, to M1a, stage IV, disease (Goldstraw et al., 2007). In spite of its frequent occurrence in clinical situation, management options have altered little over the last 80 years (Davies et al., 2013) .Thus, understanding the pathogenetic mechanisms of MPE is important and would hopefully permit the development of more specific, effective and safer treatment modalities.

Generation of new blood vessels from the preexisting microvasculature (angiogenesis) is important in MPE formation (Macchiarini et al., 1992; Bradshaw et al., 2003). Angiogenesis is regulated by a number of angiogenic and antiangiogenic cytokines.Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis and vascular permeability.It stimulates capillary formation and has specific mitogenic and chemotactic effects on vascular endothelial cells (Zebrowski et al., 1999). VEGF contributes to the formation of MPE because of their involvement in the induction of neovascularization, in vascular permeability, and in hemorrhage (Ishimoto et al., 2002). Endostatin is one of the better-characterised endogenously produced angiogenesis inhibitors, it can activate tyrosine kinase and induce endothelial cells to form various signaling complexes, thereby inducing tumor vascular endothelial cell into the process of apoptosis and inhibiting microvascular generation (O'Reilly et al., 1997; Skovseth et al., 2005). Endostatin levels in PE have been reported in only a few studies (Sumi et al., 2003; Ruiz et al., 2005). Elevated levels of endostatin in PE may represent the local production of endostatin in pleural space, a compensate response to elevated VEGF (Sumi et al., 2003).

Previous research has investigated pleural angiogenic factors and angiogenesis inhibitors determinations play a role in the diagnosis (Sack et al., 2005; Zhou et al., 2009; Koniari et al., 2011; Zhang et al., 2012), and VEGF has been reported to be correlated with a poor prognosis

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Table 1. Characteristics of the MPE Patients and Non-malignant Subjects

Characteristics		Cases (%)	Total
MPE			70
Age, yrs(range)	≥65/<65	34(48.6)/36(51.4)	
Gender	Male/Female	46(65.7)/24(34.3)	
Histology	Adenocarcinoma /Squamous cell / Small	58(82.9)/5(7.1)/7(10.0)	
Smoking status	Nonsmoker/Smoker	33(47.1)/37(52.9)	
Clinical stage	M1a/ M1b	38(54.3)/32(45.7)	
Performance status	0-1/2-4	41(58.6)/29(41.4)	
Tuberculosis patients			20
Age, y	≥65/<65	6(30)/14(70)	
Gender	Male/Female	15(75)/5(25)	

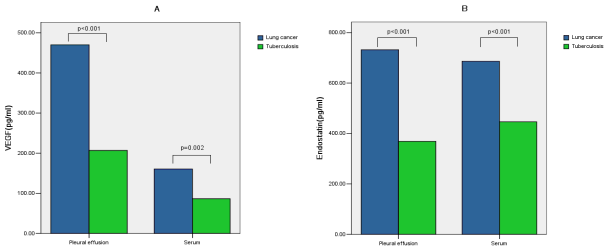

Figure 1. The Levels of VEGF (A) and Endostatin (B) for 70 Lung Cancer and 20 Tuberculosis Patients in PE and Serum. The levels of VEGF and endostatin in both PE and serum were significantly higher in lung cancer patients than those in patients with tuberculosis (by nonparametric Mann–Whitney U-test)

(Hsu et al., 2009). However, these two kinds of important factors have not yet been quantified simultaneously to determine their potential prognostic uses. Therefore, in the present study, We focused on these pro- and anti-angiogenic factors to determine the links between them and the possible role in the clinical outcomes of patient survival.

Materials and Methods

Patients

The PE and serum samples were collected from 90 patients from July 2010 to June 2013 at Nanjing Chest Hospital. The main characteristics of these patients were summarized in Table 1. This patient group included 61 men and 29 women, with a mean age of 64 years; 70 patients had MPE, while 20 had Tuberculous PE. MPE caused by lung cancer was diagnosed on the basis of malignant cells detected during cytological examination of the pleural fluid and histocytologically proven by pleural biopsy. Tuberculous PE was diagnosed with a pleural biopsy specimen showing typical epitheloid cell granuloma, and/or clinical and laboratory data suggestive of tuberculosis and response to specific antituberculous therapy. The survival time in patients with MPE was measured from the time of diagnosis to December 31, 2013 or at the date of death.

All patients signed informed consent and the study was approved by Ethics Committee of Nanjing Chest Hospital.

Sample collection and laboratory analysis

Fresh PE specimens were collected thoracoscopically before treatment.Peripheral blood samples were simultaneously obtained. Effusions and sera were collected in sterile tubes and centrifuged immediately at 4°C. Cell-free supernatants were collected and aliquots were stored at –70°C until they were analyzed.

The concentrations of VEGF (IBL, Gunma, Japan) and Endostatin (RayBiotech, Norcross, US) in PE and serum were measured with the commercially available ELISA kits according to the manufacturer's instructions.

Table 2. Multivariate Analysis of Survival in Lung Cancer Patients with MPE (by Cox's Regression)

Variable	P value	HR	95% CI for HR	
			Lower	Upper
Age	0.224	1.017	0.989	1.046
Gender	0.062	0.412	0.162	1.047
Smoking status	0.854	1.091	0.429	2.773
Histology	0.231	1.448	0.802	2.596
Clinical stage	0.736	0.900	0.489	1.657
Performance status	0.927	1.038	0.466	2.314
PE VEGF	0.015	2.152	1.163	3.605
Serum VEGF	0.520	0.843	0.508	1.426
PE Endostatin	0.032	1.860	1.102	3.918
Serum Endostatin	0.046	1.910	1.120	2.369

HR, hazard ratio; CI, confidence interval

Statistical analysis

Statistical software (SPSS for Windows, version 18) was used for the analysis. The nonparametric Mann-Whitney U-test was used to compare two groups of samples, and correlation analysis was used with the Spearman rank-order correlation. Survival analyses were conducted using the Kaplan-Meier method and survival characteristics were compared using the log-rank test, whereas the Cox proportional hazard regression model was used to compare the relative influences of different prognostic factors. $P<0.05$ was considered statistically significant.

Results

VEGF and endostatin levels in pleural effusion and serum

For both PE and serum, there were significant differences of VEGF and endostatin levels between patients with lung cancer and tuberculosis (Figure 1). The levels of VEGF and endostatin in both PE and serum were significantly higher in lung cancer patients than those in patients with tuberculosis (VEGF for PE: 470.09±281.11 and 206.89±81.67 pg/ml, $p<0.001$; for serum: 173.44±208.90 and 63.54±36.67 pg/ml, $p=0.002$.

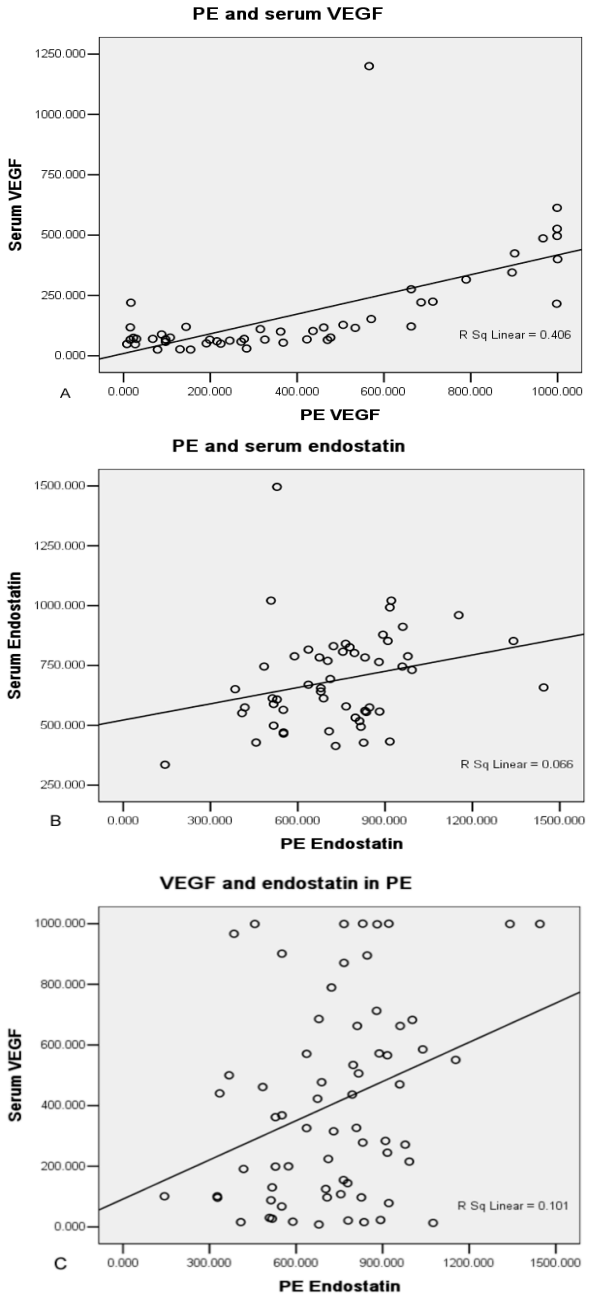

Figure 2. The significant correlations between VEGF levels in PE and serum (r=0.696, p<0.001) (**A**), endostatin levels in PE and serum (r=0.310, p=0.022) (**B**), VEGF and endostatin levels in PE (r=0.287, p=0.019) (**C**) for lung cancer patients (Spearman rank correlation)

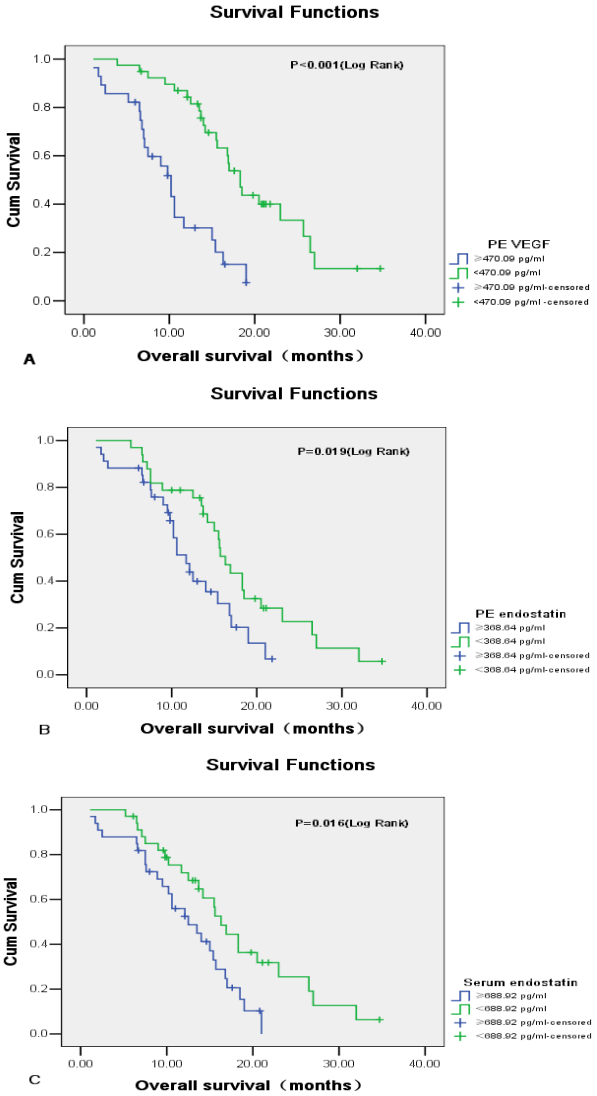

Figure 3. Kaplan–Meier survival curve for PE VEGF (A), PE endostatin (B) and serum endostatin (C). (A) Patients with PE VEGF levels<470.09 pg/ml survived longer than those who had PE VEGF ≥470.09 pg/ml (18.3 months vs. 9.8 months, P<0.001). (B) Patients who had PE endostatin levels <368.64 pg/ml survived longer than patients who had PE endostatin≥368.64 pg/ml (16.3 months vs. 11.7 months, P=0.019). (C) Patients who had serum endostatin levels <688.92 pg/ml survived longer than patients who had serum endostatin≥688.92 pg/ml (16.3 months vs. 12.5 months, P=0.016)

Endostatin for PE: 731.80±240.00 and 368.64±157.35 pg/ml, p<0.001;for serum: 688.92±202.51 pg/ml and 451.07±116.50 pg/ml, p<0.001).

Correlation of VEGF and endostatin levels between pleural effusion and serum

We examined VEGF and endostatin levels in PE and serum in 70 lung cancer patients and found the following statistically significant correlations: VEGF values in PE and serum (r=0.696, p<0.001) (Figure 2A), endostatin values in PE and serum (r=0.310, p=0.022) (Figure 2B), and VEGF and endostatin values in PE (r=0.287, p=0.019) (Figure 2C). However, no association was observed between VEGF and endostatin values in serum (p=0.257).

Survival analysis

Kaplan-Meier survival analysis was performed in the MPE group.When a VEGF value = 470.09 pg/ml in PE was used as a cut-off point (the mean value of MPE VEGF), patient's survival curve was significantly different between patients with VEGF <470.09 pg/ml and >470.09 pg/ml. Patients with PE VEGF levels <470.09 pg/ml survived longer than those who had PE VEGF >470.09 pg/ml (18.3 months vs. 9.8 months, P<0.001). (Figure 3A). Patients who had PE endostatin levels <368.64 pg/ml (the mean value of MPE endostatin) survived longer than patients who had PE endostatin >368.64 pg/ml (16.3 months vs. 11.7 months, P=0.019) (Figure 3B). In addition, patients who had serum endostatin levels <688.92 pg/ml (the mean value of serum endostatin) survived longer than patients who had serum endostatin >688.92 pg/ml (16.3 months vs. 12.5 months, P=0.016) (Figure 3C). There was no

difference between high and low serum VEGF levels for overall survival (p=0.153).

Multivariate analysis of the 70 MPE patients was carried out using the Cox regression model, which evaluated 10 factors, including age (<65 years vs≥65 years), gender, smoking status (nonsmoker vs smoker), histology (adenocarcinoma vs squamous cell carcinoma vs small cell carcinoma), clinical stage (M1a vs M1b), performance status (0-1 vs 2-4), and VEGF and endostatin levels in MPE and serum (low-level vs high-level). Independent statistically significant prognostic effects on survival were found for PE VEGF [hazard ratio (HR), 2.152; 95% confidence interval (CI), 1.163-3.605; p=0.015], PE endostatin (HR, 1.860; 95%CI, 1.102-3.918; p=0.032) and serum endostatin (HR, 1.910; 95% CI, 1.120-2.369; p=0.046).

Discussion

In the present study, we investigated an important angiogenic factor, VEGF and a potent antiangiogenic factor, endostatin in patients with tuberculosis and MPE-associated lung cancer and showed that their levels in PE and serum were significantly higher in patients with lung cancer than those in patients with tuberculosis. We also found that there were good linear correlations between VEGF levels in PE and in serum, endostatin levels in PE and in serum, and VEGF and endostatin levels in PE. More importantly, in our multivariate analysis, VEGF and endostatin in PE, and serum endostatin were revealed as independent prognostic parameters of lung cancer patients with MPE.

VEGF has been implicated as a critical cytokine in the formation of MPE. Elevated levels of VEGF produced by tumor cells, mesothelial cells, and infiltrating immune cells result in increased vascular permeability, cancer cell transmigration, and angiogenesis (Zebrowski et al., 1999; Cao et al., 2013; Dong et al., 2013; Song et al., 2013). Different authors have highlighted the role played by VEGF in the accumulation of exudative PE, especially that of MPE associated with lung cancer (Yanagawa et al., 1999). A few studies (Sack et al., 2005; Zhang et al., 2012; Zhou et al., 2009; Koniari et al., 2011; Yanagawa et al., 1999; Ziora et al., 2002; Fiorelli et al., 2011) have suggested that high VEGF levels in both serum and PE might be considered predictive of malignancy. In agreement with previous reports, the current study found that VEGF levels in PE and serum are significantly higher in malignant than in benign effusions. But VEGF was not a good discriminator because of the considerable overlap between the two groups, this was also consistent with former investigations (Yanagawa et al., 1999).

Both angiogenic factors and angiogenesis inhibitors are likely contributors to the formation of effusion in the pleural space (Ruiz et al., 2005). Endostatin is one of a number of endogenously generated antiangiogenic protein fragments that have been shown to have antitumor activity in murine models (Skovseth et al., 2005). Although serum levels of endostatin have extensively been studied in patients with malignant diseases, only a few studies evaluated endostatin levels in PE (Sumi et al., 2003;Ruiz et al., 2005; Sack et al., 2005; Zhang et al., 2012; Koniari et al., 2011; Hsu et al., 2009; Yanagawa et al., 1999; Ziora et al., 2002; Fiorelli et al., 2011; Chen et al., 2012). Previous studies reported that in comparison with either VEGF or endostatin determination in serum or pleural fluid, the combined detection of two factors improved the diagnostic sensitivity and accuracy (Zhang et al., 2012; Zhou et al., 2009). In this study, serum and pleural levels of endostatin, in accordance with VEGF, were significantly higher in malignant group than in benign group.

Variable results have been reported concerning the prognostic implications of serum VEGF levels in lung cancer. In agreement with previous studies (Laack et al., 2002; Chakra et al., 2008). we found that a high serum VEGF level did not independently determine prognosis of lung cancer.But several other studies produced conflicting results (Kaya et al., 2004; Hasegawa et al., 2005). The exact origin of VEGF in serum is still unclear. It is thought that the malignant cells themselves and inflammatory cells infiltrating tumor are the main sources of VEGF as well as peripheral blood cells like platelets and leukocytes (Salven et al., 1999; Choi et al., 2001). Serum VEGF level differed depending upon the counts of platelet and leukocyte. There is some uncertainty regarding serum VEGF levels with the survival significance, and need further investigation.

The prognostic significance of pleural VEGF in lung cancer patients with MPE has been estimated previously. Increased pleural VEGF levels are indicative of poor survival in this patient population (Hsu et al., 2009). In our study, we examined the pleural and serum VEGF levels in 70 lung cancer patients and demonstrated that there was a significant correlation between them, and the Kaplane-Meier method revealed a significant correlation between pleural VEGF levels and survival, which suggested its usefulness as a marker for estimating prognosis.

Suzuki et al (Suzuki et al., 2002) found that high levels of endostatin in serum were associated with adverse outcome in patients with NSCLC.Iizasa et al (Iizasa et al., 2004) reported that serum endostatin detected in NSCLC patients partially originates from tumor tissues. Overexpression of collagen XVIII in tumor tissue is strongly associated with a poorer outcome in NSCLC and correlates with elevated levels of circulating serum endostatin.In this study, we revealed that serum endostatin level was an independent unfavourable prognostic factor for overall survival in lung cancer patients with MPE.

To the best of our knowledge, this is the first report concerning pleural endostatin level and survival significance of MPE-associated lung cancer patients. The present study showed that pleural endostatin can serve as a prognostic factor for lung cancer patients with MPE and high pleural endostatin levels were correlated with poor overall survival outcome.Nevertheless, the mechanism underlying the association between the clinical outcome and endostatin is unclear. The elevation of the endostatin level in the high endostatin group might be a result of increased tumour angiogenesis, and endostatin levels, which reflect tumour burden.

Most importantly, endostatin levels in lung cancer patients with MPE correlated significantly with VEGF levels in PE but not in serum.The association between

endostatin and VEGF demonstrated in this study does not necessarily indicate a causal link between the elevated levels of these cytokines in this patient population. It is possible that elevated VEGF and endostatin levels may be correlated because of the homeostatic interrelationship between pro- and anti-angiogenic substances, which has been hypothesized but not fully elucidated.While elevated serum levels of VEGF and endostatin in MPE-associated lung cancer are unrelated and attributable to some clinical factors not examined in the present study. Reliable animal experiment (Ma et al., 2012) showed that Endostar, a recombinant human endostatin, played an efficient anti-cancer role in MPE through its suppressive effect on angiogenesis and lymphangiogenesis. They established mouse model of MPE and treated MPE by Endostar in high or low dose injected into pleural cavity compared with Bevacizumab, then found pleural effusion in high dose of Endostar treat group was less than Bevacizumab group, they also revealed the mechanism of Endostar, which inhibited angiogenesis and lymphangiogenesis through down-regulating the expression of VEGF-A and VEGF-C, respectively. We speculate that high dose of Endostar broke the balance of angiogenesis and antiangiogenesis, not only angiogenesis but also lymphangiogenesis were inhibited simultaneously, This was why Endostar had therapeutic effect on MPE of lung cancer and the effect was dose-dependent.

For lung cancer patients with MPE, the goals of treatment are to evacuation of the pleural fluid, prevention of reaccumulation and prolong survival (Roberts et al., 2010; Heffner et al., 2008). Some angiogenesis inhibitors such as Bevacizumab, a recombinant humanized monoclonal antibody to VEGF, and Endostar, a modified and recombinant human endostatin, either intravenously or intrapleurally, when combined with chemotherapy, have been shown to be efficient in suppressing the accumulation of pleural fluid (Hama et al., 2011; Kitamura et al., 2013; Chen et al., 2012; Du et al., 2013). reported that VEGF levels in MPE could serve as a prognostic marker for bevacizumab therapy. Whether measurement of these pro- and anti-angiogenic, tumor derived cytokines can guide treatment need more in-depth studies. We believe that elucidating the nature of the homeostatic relationship between them will be critical in providing individualized treatment strategies.

In conclusion, the overall survival of lung cancer patients with high levels of PF pro- and anti-angiogenic factors (VEGF and endostatin) is less optimistic than those with non-elevated factors. However, tumor-related angiogenesis is a dynamic process, and the clinical significance of prognostic factors at any given time may be influenced by co-factors such as EGFR mutation status, performance status, and ongoing treatment. To identify the most reliable predictors of clinical outcome will require multivariate analysis of a larger group of patients with more detailed prospective data collection.

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