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ORIGINAL ARTICLE

Diagnostic Values of sVEGFR-1 and Endostatin in Malignant Pleural Effusions in Patients with Lung Cancer

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SUMMARY

Background: **The diagnosis of malignant pleural effusion (MPE) remains a common clinical challenge because of the sensitivity of conventional cytology for the detection is insufficient. Thus, a sensitive clinical marker for diagnosis is required. The aim of this study was to assess the role of two anti-angiogenic cytokines, soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) and endostatin, in diagnosing MPE.**

Methods: **Effusion samples from 44 patients with MPE caused by lung cancer and from 36 patients with benign pleural effusion (BPE) were collected. The concentrations of sVEGFR-1 and endostatin in pleural fluid were determined by an enzyme-linked immunosorbent assay (ELISA). The diagnostic performance was measured by receiver operating characteristic curves (ROCs).**

Results: **The levels of sVEGFR-1 and endostatin in MPE due to lung cancer were significantly higher than those in BPE ($p < 0.05$). The sensitivity and specificity of endostatin were 52.27% and 86.11%, respectively, while for sVEGFR-1, the sensitivity was 88.64% and the specificity was 58.33%. Interestingly, the combination of sVEGFR-1 and endostatin produced better sensitivity and specificity of 72.73% and 83.33%, respectively. In addition, the levels of sVEGFR-1 and endostatin were significantly related to each other ($p < 0.05$), and the levels of endostatin in bloody effusions were significantly higher than those in non-bloody effusions ($p < 0.05$).**

Conclusions: **Our study indicated that the levels of sVEGFR-1 and endostatin were significantly elevated in MPE. The combined detection of sVEGFR-1 and endostatin may be useful in the diagnosis of MPE.**

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KEY WORDS

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INTRODUCTION

Malignant pleural effusion (MPE) occurs in over 175,000 patients each year, and it is a frequent complication of various malignancies. Lung cancer is the major cause [1]. Approximately 15% of lung cancer patients have an MPE at presentation and an additional 50% develop pleural effusion (PE) later in the process of their disease. MPE also implies an end stage disease and a dismal prognosis with a median survival time of approximately 4 - 6 months [2,3]. Therefore, the differentiation of malignant from benign effusions is impor-

tant for receiving timely treatment and improving prognoses. However, this remains a major clinical challenge. Cytology is the most specific, rapid, and minimally invasive diagnostic method, but it has a sensitivity of only 60%, which is insufficient for clinical needs [4]. Thoracoscopy achieves a diagnosis with approximately 95% accuracy, but it may not be adoptable in all hospitals. Moreover, it is often too invasive for patients in poor physical condition [5]. Recently, with the development of molecular biology in cancer, especially in the mechanism of tumor angiogenesis, tumor markers are widely used for the identification of PE. Therefore, we should identify new and less invasive biomarkers for the differentiation of MPE.

Angiogenesis plays a principal role in the formation of MPE [6] and is regulated by the quantity of angiogenic cytokines and anti-angiogenic cytokines. Many known angiogenic cytokines, including VEGF and matrix metalloproteinase-9, are present in the pleural fluid and may serve as diagnostic and prognostic biomarkers for MPE [7-9]. The relative levels of anti-angiogenic cytokines, such as sVEGFR-1 and endostatin in PE, have been reported in only a few studies [7,9-12]. To our knowledge, this is the first study focused on these two anti-angiogenic cytokines (sVEGFR-1 and endostatin) in pleural fluid to evaluate their diagnostic value for MPE and the possible links between them.

A VEGF-specific tyrosine kinase receptor, VEGFR-1, is an intermediary of angiogenesis in malignancy and is involved in cancer growth and metastasis [13]. A soluble form of VEGFR-1 (sVEGFR-1) has been detected in the circulation. sVEGFR-1 is a critical inhibitor of VEGF activity and thus plays a key role in tumor angiogenesis as an endogenous anti-angiogenic cytokine [14, 15]. Clinical observations have demonstrated that the pleural levels of sVEGFR-1 might be helpful for MPE diagnosis [7,10,12].

Endostatin is characterized as an efficient endogenously produced inhibitor of angiogenesis and is associated with malignant tumor development, invasion and metastasis [16,17]. Serum endostatin levels have widely been evaluated in malignancies; however, only a few studies have investigated why pleural endostatin levels are elevated in MPE and may contribute to its diagnosis [9,11, 18-20]. Based on the important role of sVEGFR-1 and endostatin in tumor angiogenesis and pleural formation, we performed a prospective study to evaluate the usefulness of the two anti-angiogenic cytokines in the diagnosis of MPE, and we were interested in determining whether there was a correlation between them.

MATERIALS AND METHODS

Patients

Between April 2014 and November 2016, a total of 80 patients (57 males and 23 females, 55.75 ± 17.23 years of age) with PE referred to our university's affiliated tertiary teaching hospital were recruited. The main characteristics of these patients are summarized in Table 1. PE patients were divided into MPE (n = 44) and BPE (n = 36) groups. MPE was diagnosed by malignant cells detected in pleural fluid or pleural biopsy specimens. The BPE patients included 29 patients with tuberculosis PE, 2 patients with parapneumonic PE and 5 patients with heart failure PE. Tuberculosis PE was diagnosed according to the following criteria: histological evidence of granulomatous tissue in pleural biopsies, microbiological proof in sputum or effusion fluid and positive response to anti-tuberculosis treatment. The diagnosis of parapneumonic PE was based on non-empyemic effusion associated with pneumonia. Heart failure PE was diagnosed with a type of transudative effusion, clinical symptoms and signs, and systolic or diastolic dysfunction of the left ventricle.

The study was approved by the Ethics Committee of Anhui Medical University and informed consent was obtained from all subjects.

Collection of samples

Fresh PE samples were obtained by diagnostic thoracentesis before any treatment. Some samples were immediately sent to the hospital laboratory for routine biochemical analysis, including total and differential cell counts, glucose, total protein (Pro), lactate dehydrogenase (LDH), PH, adenosine deaminase (ADA), carcinoembryonic antigen (CEA), and cytological examination. Other samples were centrifuged at 1500 x g for 10 minutes at 4°C, and the supernatants were immediately frozen at -80°C until the ELISA was performed.

Measurements of sVEGFR-1 and endostatin

The levels of sVEGFR-1 (ng/mL) and endostatin (ng/mL) in PE were measured using a commercially available ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's guidelines. The minimum detectable levels for sVEGFR-1 and endostatin were less than 0.156 ng/mL and 7.8 ng/mL, respectively. The clinical data of patients were blinded to the investigators.

Statistical analysis

The characteristics of patients and pleural fluid are presented as the mean ± standard deviation (SD) or medians (with interquartile ranges). The differences between MPE and BPE were analyzed using an independent Student's *t*-test or nonparametric Mann-Whitney test for measurement data and the χ^2 test for numeration data. For correlations between variables, we used Pearson's correlation coefficients. The diagnostic performance of each cytokine to differentiate MPE from BPE was analyzed with ROC analysis. The best cutoff point was selected, and sensitivity, specificity, and accuracy were calculated based on that point. SPSS 18.0 (Chicago, IL, USA) software was used for statistical analysis. p-values less than 0.05 were considered statistically significant.

Table 1. The characteristics of the patients.

	MPE (n = 44)	BPE (n = 36)	t/χ^2	p
Age, years	63.36 ± 11.80	46.44 ± 18.37	4.780	< 0.001
Gender, number			2.770	0.096
Male	28	29		
Female	16	7		
Smoking status			2.251	0.134
Smokers	22	12		
Non-smokers	22	24		
Pack/years	44.23 ± 35.17	25.3 ± 18.78	1.731	0.093
Main symptoms			0.934	0.627
Cough	26	24		
Chest distress	13	11		
Other	5	2		
Histological type				
Squamous cell carcinoma	9	-	-	-
Adenocarcinoma	31	-	-	-
Small-cell lung cancer	4	-	-	-
Benign etiology				
Tuberculosis	-	29	-	-
Bacterial pneumonia	-	2	-	-
Heart failure	-	5	-	-

Table 2. Pleural cell count and biochemical parameters in MPE and BPE.

	MPE (n = 44)	BPE (n = 36)	$t/z/\chi^2$	p
RBC/mm^3	5000.00 (1500.00 - 20175.00)	3700.00 (1950.00 - 6025.00)	-0.885	0.376
WBC/mm^3	1423.00 (990.25 - 2359.75)	2604.00 (1146.50 - 4030.75)	-2.737	0.006
Neutrophils (%)	15.85 (11.08 - 26.08)	11.70 (4.13 - 18.03)	-2.118	0.034
Mononuclear (%)	84.15 (73.93 - 88.93)	88.30 (81.97 - 95.87)	-2.118	0.034
Pro (g/L)	41.60 ± 9.86	45.01 ± 7.33	-1.720	0.089
LDH (IU/L)	868.50 (583.25)	1046.00 (513.25 - 2149.25)	-0.440	0.660
Glucose (mmol/L)	5.12 ± 1.79	6.33 ± 2.35	-2.621	0.011
PH	7.24 ± 0.22	7.20 ± 0.17	0.894	0.374
Pro pleural/serum ratio	0.64 (0.59 - 0.74)	0.69 (0.64 - 0.76)	-1.644	0.100
LDH pleural/serum ratio	4.32 (2.51 - 8.85)	5.91 (2.90 - 9.26)	-0.841	0.400
ADA (u/L)	7.80 (5.10 - 10.10)	41.60 (25.25 - 50.75)	-6.683	< 0.001
CEA (ng/mL)	63.50 (7.85 - 266.95)	0.90 (0.40 - 1.30)	-6.509	< 0.001
Cytology [a] number positive	28/44 (63%)	-	-	-
Bloody [b]	13/44 (29.5%)	4/36 (11.1%)	4.021	0.045
sVEGFR-1 (ng/mL)	7.02 ± 2.80	4.04 ± 2.40	-4.449	< 0.001
Endostatin (ng/mL)	84.12 (47.17 - 135.79)	48.70 (24.11 - 74.28)	-3.525	< 0.001

[a] - results here include those of the initial and repeated examination of cytology, if any.
[b] - bloody effusion containing more than 1 x 10^4/μL RBCs.

RESULTS

The main characteristics and different etiologies of the patients are summarized in Table 1. There were 44 lung cancer patients with MPE (28 males and 16 females; 63.36 ± 11.80 years old) and 36 patients with BPE (29 males and 7 females; 46.44 ± 18.37 years old). In the 44 lung cancer patients with MPE, the pathologic types included adenocarcinomas (n = 31), squamous cell carcinomas (n = 9), and small cell carcinomas (n = 4). There were no statistically significant differences in gender and clinical symptoms between the MPE and BPE groups (both $p > 0.05$). The age of patients with MPE was significantly higher than that of patients with BPE ($p < 0.001$). The percentage of smokers in lung cancer patients with MPE appeared higher in comparison to patients with BPE. However, there were no statistically significant differences ($p > 0.05$). This might be because smoking is more strongly linked with squamous-cell carcinoma and small-cell lung carcinoma than lung adenocarcinoma; however, adenocarcinoma is responsible for the most frequent histology of malignant pleural effusion [21].

The laboratory characteristics of the pleural fluid are summarized in Table 2. There were no significant differences in pleural protein, LDH, PH, Pro Pleural/Serum ratio, LDH Pleural/Serum ratio, and red blood cell (RBC) levels between MPE and BPE. The percentage of mononuclear cell counts was higher in MPE ($p < 0.05$). BPE exhibited a higher percentage of neutrophils and lower levels of glucose ($p < 0.05$). The levels of CEA were significantly higher in MPE ($p < 0.001$). In contrast, the levels of ADA were significantly elevated in patients with BPE compared with patients with MPE ($p < 0.001$). Pleural fluid cytology was performed with a positive rate of 63% in MPE groups.

As shown in Table 2 and Figure 1a and b, the levels of sVEGFR-1 and endostatin in MPE were significantly higher than those in BPE ($p < 0.05$).

In our study, the percentage of bloody effusions (containing more than 1 x 10^4/μL RBCs) in patients with MPE was higher than that in patients with BPE ($p < 0.05$) (Table 2). The concentrations of anti-angiogenic cytokines in bloody effusions and non-bloody (containing less x 10^4/μL RBCs) effusions were compared. Interestingly, pleural fluid endostatin concentrations in bloody effusions were significantly higher than those in non-bloody effusions ($p < 0.05$). However, the sVEGFR-1 levels showed no significant difference between bloody effusions and non-bloody effusions ($p > 0.05$) (Figure 1c and d).

ROC curves for sVEGFR-1 and endostatin were presented in Figure 2. Both the measurement of sVEGFR-1 and endostatin revealed a significant advantage in diagnosing MPE with an area under the curve (AUC) of 0.79 and 0.73, respectively ($p < 0.05$). The best cutoff value for endostatin in diagnosing MPE was 79.32 ng/mL, which yielded a sensitivity of 52.27% and specificity of 86.11%. For sVEGFR-1, the sensitivity and specificity were 88.64% and 58.33%, respectively, with a cutoff point of 3.95 ng/mL. To increase the definitiveness of the diagnosis, we chose a cutoff value of 6.36 ng/mL, with a specificity up to 80.56% and sensitivity of 56.82%. The combined detection of sVEGFR-1 and endostatin had better sensitivity and specificity of 72.73% and 83.33%, respectively, with an increased AUC of 0.84 (Figure 2).

Of the investigated laboratory parameters of the pleural fluid, the level of endostatin was positively correlated with RBC ($r = 0.387$, $p < 0.001$) and negatively correlated with ADA ($r = -0.312$, $p = 0.005$) (Figure 3a and b). The concentration of sVEGFR-1 showed significant negative correlations with ADA ($r = -0.292$, $p = 0.009$) (Figure 3c). We further found that the levels of sVEGFR-1 were significantly correlated with those of endostatin ($r = 0.297$; $p = 0.007$) (Figure 3d).

DISCUSSION

sVEGFR-1 is responsible for the pathogenesis of several diseases. Most clinical studies have focused on its mechanism in human preeclampsia [22]. Recently, the role of sVEGFR-1 as a diagnostic or prognostic marker in human cancers has garnered increasing interest [23]. However, only a few studies have investigated its role in pleural effusions [7,10,12]. Fiorelli et al. [7] detected increased levels of sVEGFR-1 in MPE indicating a possible valuable for diagnosis. Moreover, Hooper et al. [10] suggested that increased sVEGFR-1 levels in MPE were significantly related to poor prognosis.

The results of the present study demonstrate that the level of sVEGFR-1 is significantly higher in MPE associated with lung cancer than that in BPE. This observation is consistent with previous studies [7,10]. Although the high expression of sVEGFR-1 has been demonstrated in MPE; its biological role or molecular mechanisms are poorly understood. Tumor growth and metastasis are dependent on the development of angiogenesis. VEGF increases vessel permeability and plays a critical role in the accumulation of malignant effusions [24]. The formation of MPE is dependent on the invasion of the pleura by tumor cells and the expression of high levels of VEGF [25]. However, sVEGFR-1 is believed to be an endogenous inhibitor of VEGF activity [14,15]. Thus, the changes of sVEGFR-1 in MPE may reflect a compensatory response to the overexpression of VEGF levels.

Endostatin is also a potent inhibitor of angiogenesis and tumor growth. Tian et al. [18] reported that endostatin levels were significantly elevated in MPE than in BPE and pleural fluid endostatin > 79.7 ng/mL was indicative of malignancy. Similar findings were also shown by other investigators [9,19]. In the present study, we found that endostatin levels were significantly higher in MPE due to lung cancer than in BPE, and the cutoff value for diagnosing MPE was 79.32 ng/mL. This observation is in agreement with the findings of Sumi

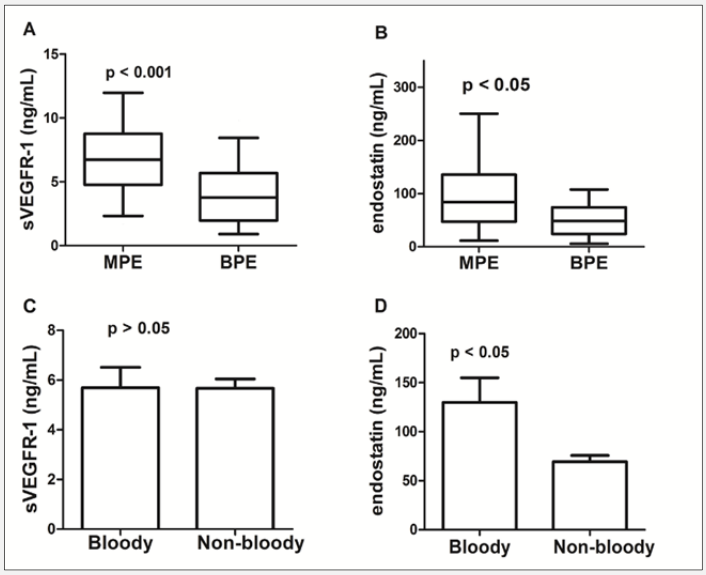

Figure 1. Comparison of sVEGFR-1 and endostatin levels between MPE and BPE.

The levels of sVEGFR-1 were significantly higher in pleural effusions of malignant than of benign diseases ($p < 0.001$) (a); a significant difference of endostatin levels was also found between the MPE and BPE ($p < 0.05$) (b). The concentrations of sVEGFR-1 and endostatin in bloody effusions and non-bloody pleural effusions were compared. There was no significant difference in the level of sVEGFR-1 between bloody effusions and non-bloody effusions ($p > 0.05$) (c). The levels of endostatin in bloody effusions were significantly higher than those in non-bloody effusions ($p < 0.05$) (d).

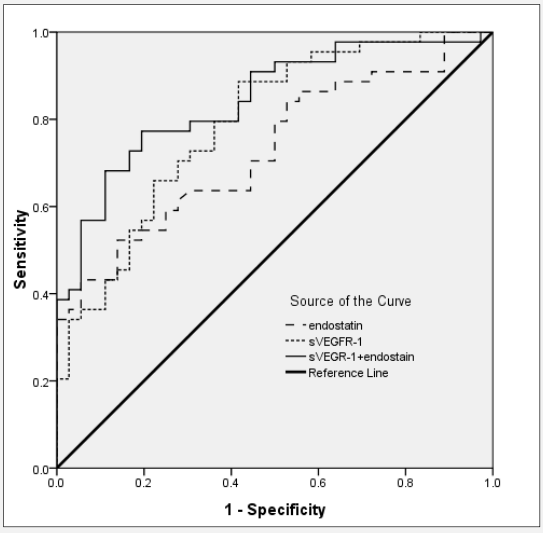

Figure 2. sVEGFR-1 reached a sensitivity of 88.64% and a specificity of 58.33% (cutoff value: 3.95 ng/mL, AUC: 0.79); endostatin reached a sensitivity of 52.27% and a specificity of 86.11% (cutoff value: 79.32 ng/mL, AUC: 0.73) in distinguishing MPE from BPE.

The combined detection of sVEGFR-1 and endostatin had better sensitivity and specificity of 72.73% and 83.33%, respectively, with an increased AUC of 0.84.

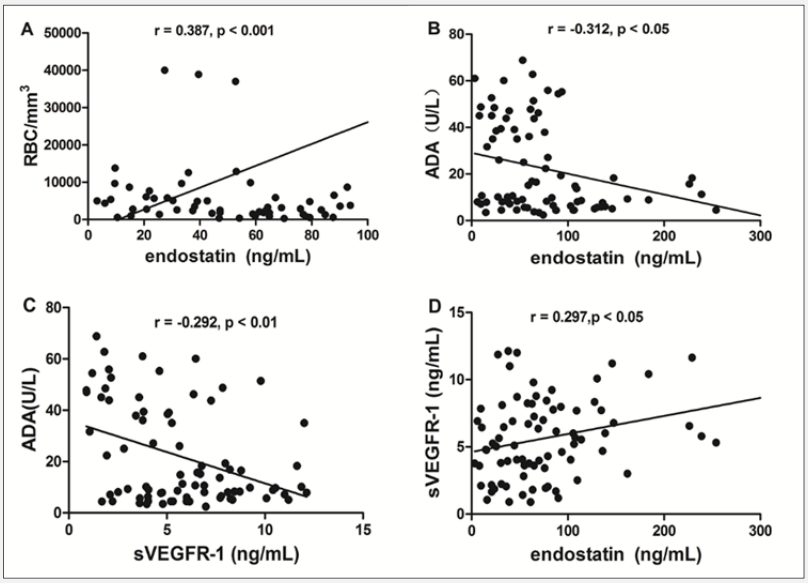

Figure 3. In the pleural fluids, (a) the level of endostatin was positively correlated with RBC (r = 0.387, p < 0.001), and (b) negatively correlated with ADA (r = -0.312, p < 0.05), (c) The concentration of sVEGFR-1 showed significant negative correlations with ADA (r = -0.292, p < 0.01), (d) The levels of sVEGFR-1 were significantly correlated with those of endostatin (r = 0.297; p < 0.05).

Et al. and Tian et al. [11,18]. The increased levels of endostatin in MPE may reflect increased tumor angiogenesis and a compensatory response to elevated VEGF levels [19].

This study indicates that the endostatin had a sensitivity of 52.27% and specificity of 86.11% in diagnosing MPE. The results are in accordance with previous published studies [18]. For sVEGFR-1, ROC curve analysis suggests that 3.95 ng/mL yields the best accuracy (75%) with a high sensitivity of 88.64% and specificity of 58.33% in diagnosing MPE. Of the few previous studies, only Fiorelli et al. [7] analyzed the diagnostic utility of pleural sVEGFR-1 and provided a higher sensitivity of 92% and specificity of 93% in diagnosing MPE. The difference may be due to the recruitment of more patients with MPE than those with BPE (73% vs. 27%, respectively) and the different origins of the malignancies. Because of the relatively low sensitivity of endostatin and the low specificity of sVEGFR-1, their clinical usefulness may be limited as diagnostic tools for MPE. However, the combination of sVEGFR-1 and endostatin produced better sensitivity and specificity of 72.73% and 83.33%, respectively, with an increased AUC of 0.84. We conclude that the combined use of sVEGFR-1 and endostatin in the diagnosis of MPE is more valuable.

Interestingly, we found both sVEGFR-1 and endostatin to have an inverse relationship with pleural ADA. High levels of ADA are widely applied in the diagnosis of tuberculosis pleural fluid [26]. Nevertheless, the biochemical relationship between ADA and MPE is far from clear [27]. Verma et al. [28] demonstrated that the serum LDH and pleural fluid ADA ratio is capable of predicting MPE. However, the simple observation of a significant correlation between endostatin and ADA is insufficient evidence for a specific role in the pathogenesis, and further studies are needed.

This is the first study to show that there is a significant positive correlation between sVEGFR-1 and endostatin concentrations in pleural effusions, indicating that the two cytokines might play essential roles in pleural effusion. Recent publications have shown that endostatin inhibits angiogenesis by directly interacting with VEGFR-2 and blocking VEGF-mediated signaling [29]. Furthermore, Olsson et al. [30] demonstrated that the anti-angiogenesis function of sVEGFR-1 was achieved by dampening angiogenic VEGF-VEGFR-2 signaling. Similar anti-angiogenesis mechanisms may contribute to the correlation between sVEGFR-1 and endostatin, which has been hypothesized but not completely clarified.

In addition, there was a strong association between pleural fluid red cell counts and endostatin levels. Additionally, endostatin concentrations in bloody effusions were significantly higher than those in non-bloody effusions, suggesting that endostatin may have an important

role in the pathogenesis of bloody effusions. However, there was no correlation between sVEGFR-1 and bloody effusions. Bloody pleural effusions are observed in both benign and malignant effusions, frequently in MPE. Vascular wall rupture or the leakage of blood cells is crucial in the accumulation of bloody pleural effusion. VEGF can increase capillary permeability, which might induce bloody pleural effusion [31]. Because endostatin is a potent inhibitor of VEGF, the level of endostatin may be elevated due to the high level of VEGF activity. Therefore, we hypothesized that increased endostatin levels in bloody effusions result from the homeostatic regulation between angiogenic and anti-angiogenic cytokines. More investigations are required to test this hypothesis.

CONCLUSION

Pleural fluid levels of sVEGFR-1 and endostatin are higher in MPE due to lung cancer than in BPE, indicating that high pleural levels are suggestive of MPE. The combined detection of sVEGFR-1 and endostatin had better sensitivity and specificity and may be useful in the diagnosis of MPE. Furthermore, there is a significant correlation between sVEGFR-1 and endostatin concentrations in pleural effusions. These findings may be important to clarify the role of anti-angiogenic cytokines in pleural effusions and merit further investigation.

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Compliance with Ethical Standards:
This study was approved by Ethics Committee of Anhui Medical University and informed consent was obtained from all the patients.

Declaration of Interest:
None declared.

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