Vascular endothelial growth factor and protein level in pleural effusion for differentiating malignant from benign pleural effusion

DA-WEI WU[1,2*], WEI-AN CHANG[1,2*], KUAN-TING LIU[1,3,4], MENG-CHI YEN[3] and PO-LIN KUO[1,5]

[1]Graduate Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University; [2]Division of Pulmonary and Critical Care Medicine; [3]Department of Emergency Medicine, Kaohsiung Medical University Hospital; [4]School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807; [5]Institute of Medical Science and Technology, National Sun Yat-Sen University, Kaohsiung 804, Taiwan, R.O.C.

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Abstract. Pleural effusion is associated with multiple benign and malignant conditions. Currently no biomarkers differentiate malignant pleural effusion (MPE) and benign pleural effusion (BPE) sensitively and specifically. The present study identified a novel combination of biomarkers in pleural effusion for differentiating MPE from BPE by enrolling 75 patients, 34 with BPE and 41 with MPE. The levels of lactate dehydrogenase, glucose, protein, and total cell, neutrophil, monocyte and lymphocyte counts in the pleural effusion were measured. The concentrations of interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor-α, interferon γ, transforming growth factor-β1, colony stimulating factor 2, monocyte chemoattractant protein-1 and vascular endothelial growth factor (VEGF) were detected using cytometric bead arrays. Protein and VEGF levels differed significantly between patients with BPE and those with MPE. The optimal cutoff value of VEGF and protein was 214 pg/ml and 3.35 g/dl respectively, according to the receiver operating characteristic curve. A combination of VEGF >214 pg/ml and protein >3.35 g/dl in pleural effusion presented a sensitivity of 92.6% and an accuracy of 78.6% for MPE, but was not associated with a decreased survival rate. These results suggested that this novel combination strategy may provide useful biomarkers for predicting MPE and facilitating early diagnosis.

Correspondence to: Dr Meng-Chi Yen, Department of Emergency Medicine, Kaohsiung Medical University Hospital, 100 Tz-You 1st Road, Kaohsiung 807, Taiwan, R.O.C.
E-mail: 1030605@ms.kmuh.org.tw

Professor Po-Lin Kuo, Graduate Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, R.O.C.
E-mail: kuopolin@seed.net.tw

*Contributed equally

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Introduction

Pleural effusion is a common clinical complication associated with multiple benign and malignant conditions (1). Congestive heart failure, pneumonia and tuberculosis are common causes of benign pleural effusion (BPE) (2). Certain types of malignancy, including lung, breast, ovarian cancer and lymphoma also cause pleural effusion (3-5). Among patients with cancer, ~50% develop malignant pleural effusion (MPE) (6). The median survival time following MPE presentation is 4 months (7).

MPE is induced by the interaction of tumor cells, endothelial cells, myeloid cells and lymphoid cells in the pleural cavity. The concentration of protein and lactate dehydrogenase (LDH) in MPE is a prognostically significant biochemical feature (8). In addition, numerous types of cytokine, including interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, IL-12, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) are detected in MPE (9-11). An increased concentration of vascular endothelial growth factor (VEGF), which is mainly secreted from endothelial cells is also detected in MPE (12). Certain biochemical properties of pleural effusion, including glucose and total protein concentration, may predict mortality in patients with MPE (13). Interferon γ (IFNG) and transforming growth factor β (TGFB) 1 are associated with tuberculosis pleural effusion, but not MPE (14,15). However, no cytokines or biochemical features that differentiate MPE and BPE sensitively and specifically have been identified at present.

Since clinical features, biomedical features and numerous cytokines have been reported to be associated with MPE, and a single parameter may not serve as an optimal biomarker for predicting MPE (8-15), the present study assessed whether a combination of biochemical features, clinical features and cytokine levels in pleural effusion may distinguish between BPE and MPE. The clinical and biochemical features of pleural effusion were determined and the concentration of cytokines in collected BPE and MPE samples was evaluated using cytometric bead arrays.

Materials and methods

Patients. In the present study, 75 patients, including 34 with BPE (22 males and 12 females; median age, 67.59 years)

and 41 patients with MPE (19 males and 22 females; median age, 65.68 years) were enrolled between January 2013 and December 2013 at the Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). The Institutional Review Board (IRB) of Kaohsiung Medical University Hospital approved the present study and all patients provided written, informed consent in accordance with the Declaration of Helsinki (IRB no. KMUH-IRB-20120275). Pleural effusion was subsequently collected. Exudative and transudative BPE was classified according to Light's criteria (16). The histology of specimens, obtained using bronchoscopy and lung puncture, or the malignant cells in the pleural effusion were used for malignancy diagnosis (17,18). MPE was collected from patients, including those with malignant tumors.

Cytometric bead array (CBA) to assess cytokine levels. Aliquots (200 μl) of pleural effusion from the 75 patients were centrifuged for 10 min at 3,000 x g at 4˚C and the supernatants were stored at -80˚C. The concentrations of IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFNG, colony stimulating factor 2, MCP-1, TNF-α, TGFB1 and VEGF were measured using a CBA Flex Set kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Each sample was determined once. Data were obtained using a BD Accuri C6 flow cytometer and analyzed using FCAP Array V3.0 software (both from BD Biosciences).

Statistical analysis. Biochemical features and the concentration of cytokines were compared between BPE and MPE samples using the Kruskal-Wallis or Mann-Whitney U test. The concentrations of cytokines and biochemical features for which these tests revealed a significant difference were used to generate a receiver operating characteristic (ROC) curve. The survival curves were obtained using the Kaplan-Meier method. SPSS version 19 (IBM Corp., Armonk, NY, USA) was used for all statistical analysis and to generate the graphs. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Causes of pleural effusion in 75 patients.

A, Patients with benign pleural effusion (n=34)

Cause of pleural effusion	No. of patients
Transudates	11
Congestive heart failure	2
Cardiogenic syncope	1
Coronary artery disease	4
Liver cirrhosis	4
Exudates	23
Bacterial pneumonia	13
Empyema	2
Pulmonary tuberculosis	6
Pleural tuberculosis	2

B, Patients with malignant pleural effusion (n=41)

Cause of pleural effusion	No. of patients
Lung cancer	28
Adenocarcinoma	23
Squamous cell carcinoma	3
Bronchogenic carcinoma	2
Extrathoracic cancer	13
Breast cancer	5
Colorectal cancer	2
Hepatocellular carcinoma	1
Esophageal cancer	1
Thyroid cancer	1
Oral cancer	1
Tongue cancer	1
Ovarian cancer	1

Results

Patient characteristics. Of the 75 patients enrolled in the present study, 41 (19 males and 22 females; median age, 65.68 years) exhibited lung cancer (adenocarcinoma, squamous cell or bronchogenic carcinoma, 28 patients) or extrathoracic cancer-induced MPE (including breast and colorectal cancer, 13 patients). The remaining 34 patients (22 males and 12 females; median age, 67.59 years) exhibited transudate (11 patients) or exudate-induced BPE (23 patients). The causes of pleural effusion are presented in Table I.

Biochemical and clinical features of MPE and BPE. Patients with BPE were divided into transudate and exudate groups, while patients with MPE were divided into lung and extrathoracic cancer groups. The levels of LDH, glucose and protein and the number of total cells, neutrophils, lymphocytes and monocytes among four groups (transudate, exudate, lung cancer and extrathoracic groups) are presented in Table II. A significant difference was demonstrated in protein concentration and lymphocyte number among the four groups (P=0.0001 and P=0.040, respectively). However, protein concentration was the only factor for which a significant difference between the BPE and MPE groups was demonstrated (P=0.007). No significant difference was observed between the level of LDH, glucose, count of total cell, neutrophil, lymphocytes and monocytes between the entire BPE and entire MPE groups (P=0.310, 0.117, 0.699, 0.840, 0.589 and 0.333, respectively). This result suggested that protein concentration but not lymphocyte number, may serve as a predictor for distinguishing between BPE and MPE.

Cytokine concentration in MPE and BPE. The concentration of cytokines was analyzed using a CBA Flex Set kit (Table III). The concentration of TNF-α (P=0.035), VEGF (P=0.002) and IFNG (P=0.020) differed significantly across the four groups. The highest concentration of IFNG was detected in the exudate group and the highest concentration of TNF-α was detected in the extrathoracic cancer group. However, neither IFNG nor TNF-α distinguished BPE and MPE; there was no significant difference between the BPE and MPE groups in the concentration of TGFB1 (P=0.865), TNF-α (P=0.589), CSF-2 (P=0.814), IFNG (P=0.321), IL-1B (P=0.594), IL-4

Table II. Biochemical and clinical characteristics of 75 patients with pleural effusion.

A, Clinical characteristics of 75 patients with pleural effusion

Characteristics of patients	Patients with transudate-induced BPE (n=11)	Patients with exudate-induced BPE (n=23)	Patients with lung cancer-induced MPE (n=28)	Patients with extrathoracic cancer-induced MPE (n=13)	n	P-value
Age, years[a]	66.36 (12.96)	68.17 (16.85)	67.04 (13.31)	62.77 (10.63)	75	0.735
No. of males[b]	4 (36.40)	18 (78.30)	13 (46.40)	6 (46.20)	75	0.050
No. of smokers[b]	4 (36.40)	11 (47.80)	9 (32.10)	3 (23.10)	75	0.472

B, Biochemical characteristics of 75 patients with pleural effusion[c]

Component of pleural effusion	Patients with transudate-induced BPE (n=11)	Patients with exudate-induced BPE (n=23)	Patients with lung cancer-induced MPE (n=28)	Patients with extrathoracic cancer-induced MPE (n=13)	n	P-value
LDH, IU/l	6, 297.5 (137.25-440.75)	13, 158 (135.5-252)	12, 220 (156.25-308.5)	9, 223 (180.5-265.5)	40	0.342
Glucose, mg/dl	9, 131 (117.5-157)	17, 141 (108-168)	22, 117 (95.5-143.25)	8, 114 (74.75-191.5)	56	0.482
Protein, g/dl	10, 1.8 (1.30-2.13)	22, 3.45 (1.88-4.63)	26, 4.2 (3.38-4.8)	10, 4.15 (2.45-4.5)	68	0.001
Cell count, /cum	11, 270 (138-990)	22, 1436.5 (199-2743.75)	27, 1065.0 (517-1980)	11, 930 (267-2270)	71	0.194
Neutrophil (%)	11, 17% (1-45)	21, 5% (3-16)	19, 13% (2-46)	10, 4.5% (1-20.5)	61	0.788
Lymphocyte (%)	11, 22% (11-51)	21, 68% (31-83)	18, 48.5% (30.5-68.25)	10, 48% (20.50-70.75)	60	0.040
Monocyte (%)	11, 19% (8-38)	21, 7% (4-21)	19, 14% (5-19)	10, 8.5% (3.75-18.5)	61	0.206

[a]Data presented as mean ± standard deviation. [b]Data presented as n (%). [c]Data presented as n, median (interquartile range). P-values were calculated using Kruskal-Wallis analysis. BPE, benign pleural effusion; MPE, malignant pleural effusion; n, number of patients; LDH, lactic dehydrogenase; cum, cumulative.

Table III. Clinical characteristics of 75 patients with pleural effusion.

Type of cytokine	Patients with transudate-induced BPE (n=11)	Patients with exudate-induced BPE (n=23)	Patients with lung cancer-induced MPE (n=28)	Patients with extrathoracic cancer-induced MPE (n=13)	P-value
TGFB1	76.18 (53.15-550.04)	101.55 (38.30-200.43)	84.19 (53.63-247.94)	115.80 (49.70-189.76)	0.990
TNF-α	0.00 (0.00-0.85)	0.11 (0.00-4.55)	0.08 (0.00-1.77)	1.80 (0.27-10.67)	0.035
VEGF	61.94 (20.51-134.80)	84.9 (24.51-210.24)	400.16 (40.12-1324.58)	1146.79 (225.55-4987.09)	0.002
CSF2	0.11 (0.00-0.45)	0.21 (0.00-0.44)	0.00 (0.00-0.58)	0.39 (0.00-1.85)	0.339
IFNG	0.30 (0.00-0.67)	1.05 (0.53-5.51)	0.58 (0.22-1.95)	0.65 (0.20-1.12)	0.020
IL-1B	0.00 (0.00-0.00)	0.00 (0.00-0.65)	0.00 (0.00-0.008)	0.00 (0.00-0.11)	0.152
IL-4	0.00 (0.00-0.00)	0.00 (0.00-0.20)	0.00 (0.00-0.11)	0.00 (0.00-0.17)	0.380
IL-5	1.30 (0.00-4.03)	2.51 (0.47-7.14)	2.935 (0.79-8.45)	1.45 (0.74-28.20)	0.526
IL-6	5884.94 (2786.79-8844.97)	7874.02 (2858.57-16297.47)	6254.9 (2566.33-11590.90)	14540.13 (3572.61-19640.46)	0.182
IL-8	497.86 (100.66-1089.34)	311.10 (80.95-1192.00)	311.46 (105.27-1123.08)	1129.18 (199.46-4560.84)	0.343
IL-10	24.19 (10.37-62.67)	23.19 (11.82-38.65)	24.16 (14.21-34.93)	20.81 (12.74-57.15)	0.951
IL-12	0.00 (0.00-0.00)	0.00 (0.00-0.65)	0.00 (0.00-0.008)	0.00 (0.00-0.11)	0.181
MCP-1	514.37 (268.95-1852.68)	615.07 (187.60-1134.39)	549.50 (154.86-2072.26)	905.90 (457.38-9980.78)	0.419

Data presented as median (interquartile range) ng/ml. BPE, benign pleural effusion, MPE, malignant pleural effusion; n, number of patients; TGFB, transforming growth factor β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; CSF, colony stimulating factor; IFNG, interferon γ; IL, interleukin; MCP, monocyte chemoattractant protein. P-values were calculated using Kruskal-Wallis analysis.

Figure 1. Receiver operation curve analysis of the concentration of protein and VEGF in pleural effusion. VEGF, vascular endothelial growth factor.

(P=0.783), IL-5 (P=0.449), IL-6 (P=0.568), IL-8 (P=0.530), IL-10 (P=0.827), IL-12 (P=0.371) and MCP-1 (P=0.489). The results of the present study revealed that VEGF concentration in MPE was increased compared with that in BPE (P=0.001). Therefore, the present study suggests that VEGF may potentially distinguish MPE and BPE.

Identifying MPE and BPE according to protein and VEGF concentration. The ROC curve of protein and VEGF concentration was used to generate the optimal cutoff point for MPE and BPE. The protein concentration cutoff point [area under the curve (AUC): 0.708] was 3.35 g/dl and the VEGF cutoff point (AUC: 0.728) was 214 pg/ml for predicting MPE (Fig. 1). In accordance with the cutoff value of VEGF and protein, the sensitivity, specificity and accuracy of VEGF (>214 pg/ml; sensitivity, 70.6%; specificity, 82.4%; accuracy, 76.0%), protein (>3.35 g/dl; sensitivity, 75.6%; specificity, 70.6%; accuracy, 73.3%), and VEGF and protein (VEGF, >214 pg/ml; protein, >3.35 g/dl; sensitivity, 92.6%; specificity, 61.7%; accuracy, 78.6%) were presented in Table IV. However, the concentration of VEGF and protein was not associated with a poor survival rate (Fig. 2).

Discussion

The clinical and biochemical features of pleural effusion, including the level of procalcitonin, adenosine deaminase, C-reactive protein, carcinoembryonic antigen, and LDH have been demonstrated to represent diagnostic markers in differentiating MPE from tuberculosis pleural effusion (19,20). However, the present study demonstrated no significant difference in the level of LDH between BPE and MPE groups. BPE samples were collected in the present study from patients with different diseases, including 8 patients with tuberculosis, and this may have decreased the sensitivity of LDH. A previous study suggested that the ratio of serum LDH to adenosine deaminase in pleural fluid enhanced the sensitivity

Table IV. Accuracy of predictors in PE from 75 patients.

PE component and concentration	TPR (%)	FPR (%)	SPC (%)	ACC (%)	PPV (%)	NPV (%)
VEGF, 214 pg/ml	70.6	17.1	82.4	76.0	82.8	70.0
PE total protein, 3.35 g/dl	75.6	29.4	70.6	73.3	75.6	70.6
VEGF, 214 pg/ml and PE total protein, 3.35g/dl	92.6	25.5	61.7	78.6	74.5	87.5

VEGF, vascular endothelial growth factor; PE, pleural effusion; TPR, true positive rate; FPR, false positive rate; SPC, specificity; ACC, accuracy; PPV, positive predict value; NPV, negative predict value.

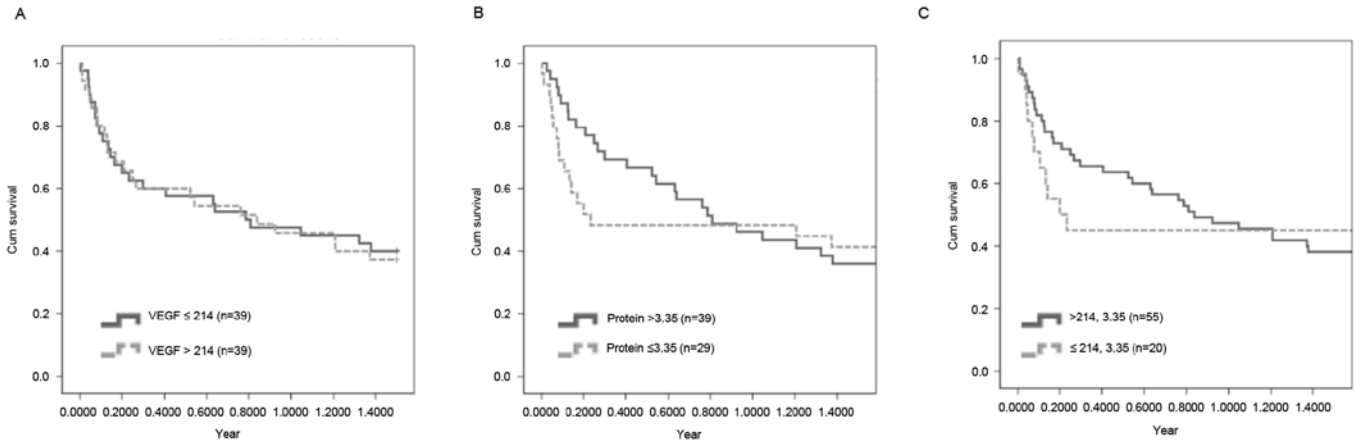

Figure 2. Kaplan-Meier survival curves for the overall cum survival rate according to the concentration of m (A) VEGF, (B) protein and (C) VEGF and protein. Cum, cumulative; VEGF, vascular endothelial growth factor; n, number of patients.

and specificity for identifying MPE (21), a result that requires further study. Furthermore, protein, glucose concentration, total cell, neutrophil, monocyte and lymphocyte counts represent MPE-associated features (22,23). However, protein concentration was the only parameter to reveal a significant difference between BPE and MPE groups in the present study. Therefore, protein concentration may potentially serve to distinguish between MPE and BPE.

Although numerous types of cytokine may be detected in BPE and MPE, the pattern of cytokines may not differentiate MPE and BPE (24,25). The present study demonstrated no significant difference in the concentration of cytokines between the MPE and BPE groups, except for TNF-α, IFNG and VEGF. However, while increased concentrations of TNF-α and IFNG were observed in the extrathoracic cancer and exudates groups, respectively, there was no significant difference between MPE and BPE overall. VEGF was used as a biomarker in the present study (optimal cutoff value=214 pg/ml). Duysinx *et al* (26) suggested that the optimal value of VEGF for differentiating MPE from BPE is 382 pg/ml and Fiorelli *et al* (27) demonstrated that sensitivity and specificity were 63 and 83%, respectively, when VEGF is >652 pg/ml. These cutoff values may differ from that of the present study due to the use of different experimental designs and sample sizes.

VEGF induces vascular permeability and is a critical mediator of pleural effusion formation (28), suggesting that blocking VEGF potentially represents a strategy for suppressing the formation of pleural effusion (29). A previous study demonstrated that the level of VEGF in pleural effusion was associated with lymph node and distant metastasis and that the IL-8 level in pleural effusion was associated with lymph node metastasis (30). Due to the limitation of a small sample size, patients with MPE were not divided into patients with primary and metastatic tumors in the present study. The association between VEGF and IL-8 and metastasis as described by this previous study was not observed in MPE samples in the present study.

The combination of VEGF and protein for differentiating BPE and MPE increased the sensitivity and accuracy but decreased the specificity compared with using a single parameter. In addition, a poor survival rate was not significantly associated with VEGF, protein or the combination of the two. To the best of our knowledge, the present study is the first to use a combination of pleural effusion VEGF and protein levels to predict whether pleural effusion from patients was malignant. To conclude, this novel combination may represent a tool for predicting MPE and facilitating early diagnosis, but not for predicting the survival rate of patients with MPE and BPE.

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