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The significance of Fas, tumor necrosis factor-related apoptosis-inducing ligand and fibrinolytic factors in the assessment of malignant pleural effusion

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Abstract: Few studies have examined the usefulness of soluble apoptotic markers for the screening of pleural effusion. This study aimed to investigate the significance of Fas, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and fibrinolytic factors for the assessment of patients with malignant pleural effusion. A total of 137 patients with pleural effusion were evaluated. Soluble Fas, TRAIL, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), D-dimers and lactate dehydrogenase (LD) levels were measured. Pleural fluid/blood (P/B) ratios of fibrinolytic factors were calculated. Fas and TRAIL levels were significantly higher in patients with malignant effusion than in those with non-malignant effusion. Malignant effusion was 1.6-fold more prevalent in patients with elevated Fas than in those without (48.5% vs 30.4%, P=0.031). The P/B ratio of tPA was 2.5-fold higher in malignant effusion than in non-malignant effusion (4.65 vs 1.83, P<0.001). Fas was positively correlated with tPA and D-dimers, but not with biochemical parameters. The ability of Fas to identify malignant effusions was significantly greater than those of tPA and LD. In conclusion, measurements of Fas and TRAIL in conjunction with fibrinolytic factors may provide information useful for monitoring patients with suspected malignant pleural effusion.

Keywords: pleural effusion; apoptosis; malignancy; fibrinolytic factor; Fas; TRAIL

INTRODUCTION

Pleural effusion is a common clinical problem that occurs as a complication of many different diseases [1]. Parapneumonic effusions are observed in nearly 40% of patients who require admission therapy for bacterial pneumonia [2]. Pleural effusion results from a disruption in pleural homeostasis and a subsequent increase in pleural permeability [3]. Many proinflammatory cytokines and the coagulation system contribute to the inflammatory process of pleural diseases, and consequently, fibrin deposition is a characteristic feature of pleural inflammation [4].

The differential diagnosis of pleural effusions is difficult. About 25% of transudates are misclassified as exudative pleural effusions when Light's criteria are used, especially in patients undergoing diuretic treatment [5]. Several biomarkers have been introduced to discriminate the causes of pleural effusions, but a considerable proportion of pleural effusions are not diagnosed [6].

Fas, also called APO-1 (CD95), is a cell surface death receptor expressed in many cells, leading to the induction of apoptosis when triggered by agonistic antibodies or the Fas ligand [7]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a transmembrane protein that can also induce apoptosis in various tumor cells. TRAIL preferentially induces apoptosis in cancer cells but not in normal cells, and thus is viewed as a potential component of cancer therapeutic strategies [8].

Apoptosis contributes to the development of pleural effusions [9]. However, there have been conflicting

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results on apoptotic activity in patients with pleural effusion. In one study, soluble Fas levels were significantly higher in malignant pleural effusion than in non-malignant pleural effusion [10]. In contrast, in another study the pleural level of an apoptotic marker was lower in malignant effusion than in non-malignant effusion [11].

In clinical practice, malignant and non-malignant effusions must be differentiated because their managements and outcomes differ. Because few studies have addressed the usefulness of soluble apoptotic markers and fibrinolytic factors for the screening of patients with pleural effusion, we undertook this study to investigate the significance of Fas, TRAIL, and biochemical and fibrinolytic parameters for assessing patients with suspected malignant pleural effusion.

MATERIALS AND METHODS

Ethics statement

This study was performed according to the guidelines of the Helsinki Declaration. The study protocol was reviewed and approved by the institutional review board, and written informed consent was obtained from all participants. All samples were collected after a sufficient explanation of the study procedure.

Subject populations

One hundred and thirty-seven patients with pleural effusion were evaluated. The subjects consisted of 92 men and 45 women with a median age of 61 years (age range 34-79 years). Only patients with exudative pleural effusion of new-onset were enrolled. Subjects with confirmed pleural exudates were chosen based on the following criteria: cytologic or histologic examination, biochemical data, radiographic findings and positive stain or culture for *Mycobacterium tuberculosis*. The primary tumors of patients with malignant pleural effusions were lung cancer (n=30), breast cancer (n=12), lymphoma (n=7), gastrointestinal carcinoma (n=3) and mesothelioma (n=2). Parapneumonic effusions refer to those associated with bacterial pneumonia. The following subjects were excluded from the study to avoid their effects on the soluble apoptotic markers and fibrinolytic factors: (i) those with a medication history including antibiotics or anti-cancer drugs; (ii) those who had undergone recent surgery; (iii) those with lung abscess, bronchiectasis and empyema. Subjects with missing values in medical records were also excluded from the analysis. Information on cigarette smoking status was obtained.

Sample collection

Pleural fluid samples were obtained using a standard thoracentesis technique at the same time as blood sampling and assessed using Light's criteria [12]. All samples were collected before starting treatment. Samples were immersed in ice and centrifuged at 1500 ×g for 15 min and immediately analyzed for routine parameters, such as pH, glucose, lactate dehydrogenase (LD), leukocyte count and its differentials, and a microbiological examination. In addition, aliquots of the supernatants were stored at -80°C until further analysis. For patients who underwent thoracentesis more than once during hospitalization, only the results of the first tap were considered.

Measurement of laboratory parameters

The concentrations of Fas, TRAIL, tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1), D-dimers, C-reactive protein (CRP) and effusion-related biochemical parameters were measured. TRAIL and Fas levels were analyzed by an enzyme-linked immunosorbent assay (ELISA) using the Quantikine human TRAIL/TNFSF10 kits (R&D Systems, Minneapolis, MN, USA) and the human sAPO-1/Fas BMS245 kits (Bender MedSystems, Vienna, Austria), respectively. D-dimers, tPA and PAI-1 levels were measured by an immunoturbidimetric assay and an enzyme immunoassay. Total protein, LD, glucose and CRP levels were quantified using a chemical analyzer (Cobas 8000 C702; Roche, Mannheim, Germany). Pleural fluid pH levels were determined using a digital pH meter (pH 310; Oakton Instruments, Vernon Hills, IL, USA). Pleural fluid/blood (P/B) ratios of biochemical and fibrinolytic parameters were calculated by dividing pleural fluid levels by respective blood levels.

Categorization of subjects

The patients were categorized into two groups according to the etiology of the pleural effusions as follows: malignant effusion (n=54) and non-malignant effusion (n=83). Patients with non-malignant effusion were subdivided into a parapneumonic effusion group (n=40) and a tuberculous effusion group (n=43). In addition, patients were further stratified into an elevated Fas group (> 473.5 pg/mL; n=68) and a non-elevated Fas group (≤473.5 pg/mL; n=69), based on the median level of Fas in patients with pleural effusion.

Statistical analysis

Data were expressed as the mean±standard deviation (SD) or median (interquartile range (IQR)). Categorical variables were presented as frequencies and percentages. Data normality was determined using the Kolmogorov-Smirnov test. Normally distributed continuous variables were analyzed using the Student's t-test, and non-normally distributed variables were analyzed using the Mann-Whitney U test. The chi-square test was used to analyze the categorical variables. Relationships between apoptotic markers and biochemical and fibrinolytic parameters were assessed by multivariate linear regression analysis with an adjustment for potential confounders, such as age, sex, body mass index (BMI), systolic blood pressure (SBP) and current smoking history. The ability of Fas, tPA and LD to detect malignant effusion was evaluated by receiver operating characteristic (ROC) curve analysis. Statistical analysis was performed using the SPSS software package (ver. 26; IBM SPSS Statistics, Armonk, NY, USA) and the MedCalc software package (ver. 20; MedCalc Software Ltd., Ostend, Belgium). Values of P<0.05 were considered statistically significant.

RESULTS

Fas and TRAIL in malignant and non-malignant effusions

Fas and TRAIL levels in malignant pleural effusions were 473.5 pg/mL and 104.0 pg/mL, which was significantly higher than those in non-malignant pleural effusions (315.0 pg/mL and 64.5 pg/mL, respectively, P<0.001) (Fig. 1). Compared to patients with non-malignant effusion, those with malignant effusion had a significantly higher tPA level and a significantly lower polymorphonuclear leukocyte count. However, there was no significant difference in the biochemical parameters between the two groups (Table 1).

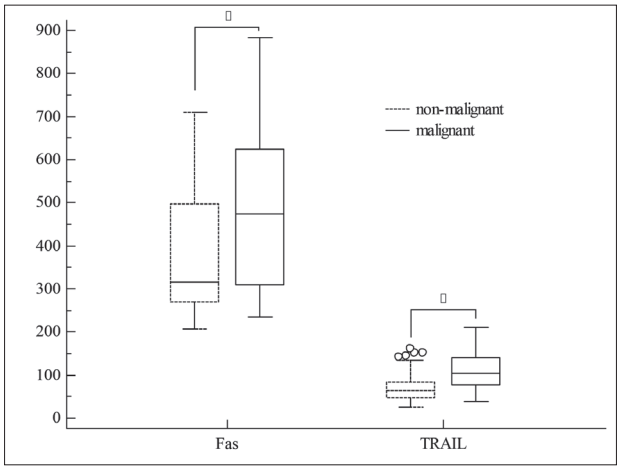

Fig. 1. Fas and TRAIL levels in patients with malignant and non-malignant pleural effusions. The levels of Fas and TRAIL are significantly higher in patients with malignant effusion than in those with non-malignant effusion (473.5 pg/mL and 104.0 pg/mL vs 315.0 pg/mL and 64.5 pg/mL, respectively). * P<0.001. TRAIL – tumor necrosis factor-related apoptosis-inducing ligand.

Tuberculous and parapneumonic effusions

Parapneumonic effusions had significantly lower pH and total protein levels than tuberculous effusions. Furthermore, PAI-1 levels were significantly higher in tuberculous effusions than in parapneumonic effusions. However, no significant difference in the Fas and TRAIL levels was observed between the two groups (Table 2).

Patients with and without elevated Fas

Malignant effusions were 1.6-fold more prevalent in patients with an elevated Fas level (48.5% vs 30.4%, P=0.031), and tPA and D-dimers levels were significantly higher in the pleural fluids of patients with an elevated Fas level. However, levels of biochemical parameters, such as LD, glucose and total protein in pleural fluid samples were not different in patients with and without an elevated Fas level (Table 3).

Table 1. Clinical and laboratory characteristics of patients with pleural effusion.

	Malignant effusion (n=54)	Non-malignant effusion (n=83)	P-value
Anthropometric parameters			
Age (year; range)	63 (38-79)	59 (34-76)	0.172
Sex (male, %)	40 (74.1)	52 (62.7)	0.164
BMI (kg/m^2)	22.8±3.7	23.1±3.9	0.651
SBP (mmHg)	128.5±17.3	129.1±17.8	0.843
Current smoking (n, %)	29 (53.7)	35 (42.1)	0.185
Biochemical parameters			
pH	7.3 (7.2-7.5)	7.1 (6.9-7.3)	0.062
LD (U/L)	1143.0 (821.3-1876.5)	1572.0 (954.5-2481.0)	0.201
Glucose (mg/dL)	65.0 (47.5-97.0)	71.5 (49.0-98.0)	0.105
Total protein (g/dL)	4.3 (2.8-5.9)	4.1 (2.4-5.3)	0.276
Inflammatory parameters			
Total leukocyte (/μL)	3152.0 (1723.0-8651.0)	6713.5 (2191.0-9281.0)	0.081
PMN (/μL)	1279.5 (617.0-2304.5)	4394.0 (1027.0-6143.5)	<0.001
Lymphocyte (/μL)	1614.0 (281.0-3072.0)	1825.2 (964.0-3748.0)	0.129
CRP (mg/dL)	2.9 (1.3-6.5)	7.1 (3.2-15.4)	<0.001
Fibrinolytic factors			
D-dimers (μg/mL)	14.2 (5.1-28.5)	9.8 (3.7-16.2)	0.102
tPA (ng/mL)	28.5 (16.7-50.3)	15.6 (12.8-41.5)	<0.001
PAI-1 (μg/L)	162.4 (89.0-190.2)	231.5 (172.3-391.6)	0.083

Data are expressed as mean ± SD, median (IQR), or frequency (%).
BMI – body mass index; SBP – systolic blood pressure; LD – lactate dehydrogenase; PMN – polymorphonuclear leukocyte; CRP – C-reactive protein; tPA – tissue-type plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1.

Table 2. Apoptotic markers and effusion-related parameters in tuberculous and parapneumonic effusions.

	Tuberculous effusion (n=43)	Parapneumonic effusion (n=40)	P-value
Apoptotic markers			
Fas (pg/mL)	328.5 (257.4-510.7)	301.6 (238.2-491.5)	0.461
TRAIL (pg/mL)	65.3 (41.5-89.1)	62.5 (37.2-82.3)	0.782
Biochemical parameters			
pH	7.5 (7.3-7.6)	6.8 (6.5-7.2)	<0.001
LD (U/L)	1239.4 (821.6-2034.7)	1826.5 (982.3-3792.6)	0.084
Glucose (mg/dL)	84.0 (51.3-96.0)	59.0 (46.5-94.5)	0.071
Total protein (g/dL)	5.2 (4.8-5.5)	3.3 (2.9-4.7)	<0.001
Inflammatory parameters			
Total leukocyte (/μL)	1824.0 (970.5-3145.0)	12145.0 (5348.5-22809.0)	<0.001
PMN (/μL)	574.0 (258.7-968.2)	8736.0 (4130.6-16725.3)	<0.001
Lymphocyte (/μL)	1283.0 (692.0-2547.0)	2253.0 (1019.5-4170.5)	0.106
CRP (mg/dL)	5.6 (1.7-9.8)	8.5 (4.2-16.3)	0.092
Fibrinolytic factors			
D-dimers (μg/mL)	8.7 (2.0-14.3)	10.6 (3.2-15.9)	0.415
tPA (ng/mL)	14.1 (11.7-40.8)	16.2 (13.1-42.5)	0.629
PAI-1 (μg/L)	302.5 (189.7-492.5)	167.2 (107.6-228.3)	0.007

Data are expressed as median (IQR).
TRAIL – tumor necrosis factor-related apoptosis-inducing ligand; LD – lactate dehydrogenase; PMN – polymorphonuclear leukocyte; CRP – C-reactive protein; tPA – tissue-type plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1.

P/B ratios of biochemical and fibrinolytic parameters

The P/B ratio of D-dimers in patients with malignant effusion was significantly above the value in those with non-malignant effusion. In particular, the P/B ratio of tPA was 2.5-fold higher in malignant effusions than in non-malignant effusions (4.65 vs 1.83, P<0.001). However, no significant difference was noted in the P/B ratio of PAI-1 and biochemical parameters between patients with and without malignant effusion (Table 4).

Relationship between Fas and TRAIL and fibrinolytic factors

After adjusting for potential confounders, Fas was positively correlated with tPA (r=0.243) and D-dimers (r=0.215). TRAIL was significantly correlated with tPA (r=0.221) but not with D-dimers. However, there was no significant correlation between apoptotic markers and biochemical parameters (Table 5). Scatter plots of the relationship between Fas and tPA are shown in Fig. 2.

Ability of Fas, tPA and LD to identify malignant effusion

The ability of Fas, tPA and LD to identify malignant effusion was assessed using ROC curve analysis. The efficacy of Fas was significantly greater than those of tPA and LD (area under the curve [AUC]: 0.755, 95% CI, 0.670-0.840 vs AUC: 0.651, 95% CI, 0.545-0.757 and 0.553, 95% CI, 0.456-0.650, respectively, P<0.001) (Fig. 3).

DISCUSSION

This study examined the soluble apoptotic markers and biochemical and fibrinolytic parameters in patients with pleural effusion. The principal findings of this study were as follows: (i) Fas and TRAIL levels

Table 3. Biochemical and fibrinolytic parameters and the prevalence of malignant effusions according to Fas level.

	Pleural Fas		
	Elevated (n=68)	**Non-elevated (n=69)**	**P-value**
Biochemical parameters			
pH	7.2 (7.1-7.3)	7.3 (7.0-7.4)	0.522
LD (U/L)	1532.4 (942.6-2793.0)	1129.3 (795.4-2407.2)	0.131
Glucose (mg/dL)	67.5 (46.0-94.5)	69.0 (45.0-97.0)	0.105
Total protein (g/dL)	4.2 (2.7-5.8)	4.3 (2.5-5.9)	0.276
Fibrinolytic factors			
D-dimers (μg/mL)	16.1 (7.4-31.5)	7.8 (4.3-19.1)	0.024
tPA (ng/mL)	37.2 (18.5-69.2)	16.4 (11.2-41.3)	0.009
PAI-1 (μg/L)	176.3 (98.3-242.7)	217.5 (102.4-357.6)	0.185
Prevalence of pleural effusions			
Malignant effusions (n, %)	33 (48.5)	21 (30.4)	0.031
Non-malignant effusions (n, %)	35 (51.5)	48 (69.6)	0.030

Data are expressed as median (IQR) or frequency (%).
LD – lactate dehydrogenase; tPA – tissue-type plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1.

Table 4. P/B ratios of biochemical and fibrinolytic parameters of patients with pleural effusion.

	P/B ratios		
	Malignant effusion (n=54)	**Non-malignant effusion (n=83)**	**P-value**
Biochemical parameters			
LD	3.28 (1.02-7.61)	3.94 (1.25-8.13)	0.451
Glucose	0.73 (0.34-1.68)	0.82 (0.36-1.95)	0.683
Total protein	0.62 (0.29-1.73)	0.65 (0.34-1.82)	0.702
Fibrinolytic factors			
tPA	4.65 (2.13-7.61)	1.83 (1.29-4.15)	< 0.001
PAI-1	4.72 (2.06-7.52)	5.06 (2.41-9.73)	0.125
D-dimers	3.91 (1.85-6.93)	1.97 (1.02-3.42)	< 0.001

Data are expressed as median (IQR).
LD – lactate dehydrogenase; tPA – tissue-type plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1; P/B ratios – pleural fluid/blood ratios.

Table 5. Relationship between apoptotic markers and biochemical and fibrinolytic parameters.

	Multivariate regression analysis* (n=137)	
	Fas (pg/mL)	**TRAIL (pg/mL)**
Biochemical parameters		
pH	0.098 (0.612)	0.092 (0.627)
LD (U/L)	0.134 (0.203)	0.127 (0.281)
Glucose (mg/dL)	0.101 (0.475)	0.103 (0.452)
Total protein (g/dL)	0.173 (0.101)	0.112 (0.324)
Fibrinolytic factors		
tPA (ng/mL)	0.243 (<0.001)	0.221 (<0.001)
PAI-I (μg/L)	-0.162 (0.108)	-0.136 (0.162)
D-dimers (μg/mL)	0.215 (<0.001)	0.158 (0.133)

Data are expressed as standardized β (p-value).
*Adjusted for age, sex, BMI, SBP and current smoking.
LD – lactate dehydrogenase; tPA – tissue-type plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1; TRAIL – tumor necrosis factor-related apoptosis-inducing ligand.

were elevated significantly in malignant effusions; (ii) Fas was positively correlated with tPA and D-dimers; (iii) Fas showed better diagnostic performance for malignant effusions than tPA and LD. These findings are in accordance with the results of a previous study [10] which demonstrated that soluble Fas levels in malignant pleural effusions were significantly higher than those in transudates and tuberculous pleural effusions. Considering that malignant effusions are a type of metastatic tumor, our results support the notion that soluble Fas can block Fas-mediated apoptosis, and thereby, Fas-expressing tumor cells can acquire the ability to escape from host immune surveillance [13,14].

Our results contrast with those of several previous studies. For instance, it has been reported that the expression of Fas was partially or completely lost in the tumor cells of malignant pleural effusions [15]. It was reported that most cases with a high percentage of apoptotic cells were tuberculous exudates, whereas only a few samples of malignant effusions showed an elevation of apoptotic activity [16]. These inconsistencies may be due to differences in detection methods between the immunohistochemical assay for Fas expression and measurements of the concentration of a truncated soluble Fas molecule. Furthermore, these discrepancies may be derived from the heterogeneity of the pathologic types of cancers in patients with malignant effusions.

TRAIL is a cytokine that selectively induces apoptosis by binding cell surface death receptors primarily in tumor cells but not in normal tissue cells [17]. TRAIL plays an important role in the regulation of tumor initiation and development [18]. One study reported that TRAIL expression is upregulated in T lymphocytes of pleural effusions [19]. In one study [20], TRAIL levels were found to be significantly higher in malignant pericardial effusions than in non-malignant pericardial effusions. In our study, TRAIL levels in malignant pleural effusions were significantly higher than those in non-malignant pleural effusions,

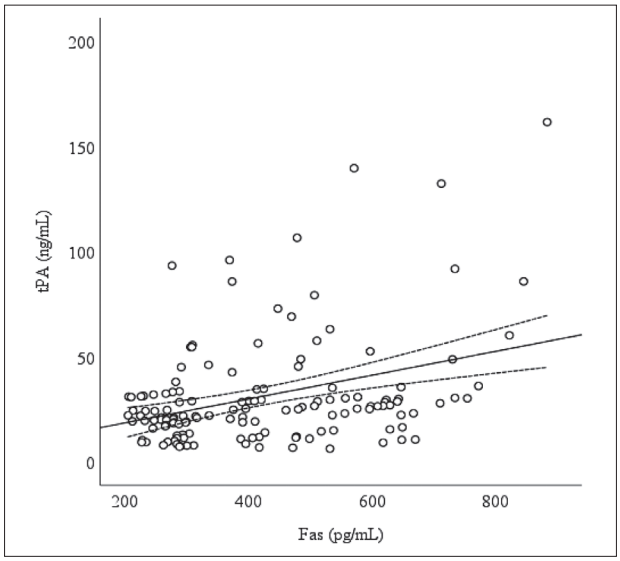

Fig. 2. Scatter plots of the relationship between Fas and tPA in patients with pleural effusion. Fas concentration is significantly and linearly correlated with tPA level ($y=0.057x+7.26$; $r^2=0.124$; $P<0.001$). tPA – tissue-type plasminogen activator.

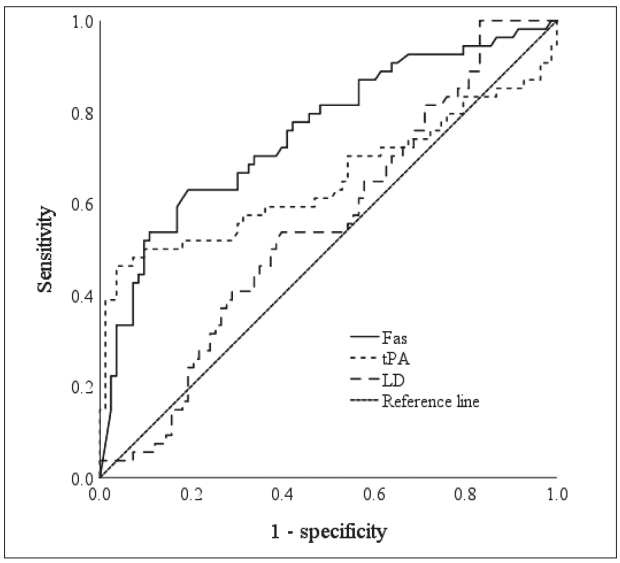

Fig. 3. ROC curves showing the ability of Fas, tPA, and LD to diagnose malignancy in pleural effusion. The area under the curve (AUC) for Fas is 0.755 (95% CI, 0.670-0.840), which is significantly larger than those for tPA and LD (AUC: 0.651; 95% CI, 0.545-0.757 and 0.553; 95% CI, 0.456-0.650; respectively, $P<0.001$). tPA – tissue-type plasminogen activator; LD – lactate dehydrogenase.

whereas TRAIL levels were similar in patients with tuberculous or parapneumonic effusions. These findings suggest that elevated TRAIL levels may be related to the development of malignant pleural effusion.

Fibrinolysis is implicated in cancer invasion by converting inactive plasminogen to active plasmin, which leads to the degradation of extracellular matrix protein in surrounding tissues [21,22]. Moreover, tPA and its inhibitor, PAI-1, play important roles in tumor growth [23,24]. PAI-1 promotes tumor cell invasion by regulating urokinase-type plasminogen activator (uPA) receptor-dependent cell adhesion. Furthermore, the presence of exogenous PAI-1 inhibits the apoptotic process [25]. In our study, patients with malignant effusion exhibited higher tPA levels than those with non-malignant effusion. tPA was significantly higher in patients with elevated Fas than in those without elevated Fas. tPA was significantly correlated with Fas and TRAIL, even after adjusting for potential confounders. These results agree with those of a previous study, showing that tPA and uPA levels were elevated in the pleural effusions of patients with cancers [26]. We believe that tumor cells in patients with malignant pleural effusion may construct a microenvironment that favors tumor cell invasion by modulating the fibrinolytic system.

Cell death is associated with impaired membrane integrity, which can be monitored by measuring LD levels in extracellular fluid [27,28]. LD is a typical intracellular enzyme that is released by cell destruction, and its release is considered a late event of apoptosis [29,30]. For example, it was reported that the induction of cell death by anti-Fas treatment resulted in a time- and dose-dependent release of LD [31]. In the present study, we assessed relationships between apoptotic markers and biochemical parameters. Multivariate regression analysis failed to reveal a significant correlation between LD and apoptotic markers, and no significant difference was found in LD levels between patients with malignant and non-malignant effusions or between patients with and without elevated Fas levels. Based on these results, LD does not seem to reflect the magnitude of apoptotic activity accurately, at least in patients with pleural effusion. These inconsistent results may be attributable to the difference between *in vitro* cell-based experiments and the examination of pleural fluids obtained from patients under different conditions.

In the present study, the P/B ratio of the fibrinolytic profiles was evaluated. There was no significant difference in the levels of pleural D-dimers between malignant and non-malignant effusions; however, a significant difference in the calculated P/B ratio for

the D-dimers was observed between the two groups. In particular, the P/B ratio of tPA was 2.5-fold higher in malignant effusions than in non-malignant effusions. These results imply that an elevated P/B ratio is not due to diffusion by increased vascular permeability, but rather due to the local production of D-dimers in the pleural cavity. Thus, the calculated P/B ratio may be more useful than the uncalculated isolated value for pleural fluid alone.

This study has several limitations. Fas and TRAIL levels could not be measured in serial samples to evaluate possible disease progression. The unmeasured confounders may have affected our results. As this investigation was a cross-sectional study, the evidence for a cause-and-effect relationship between apoptotic markers and malignant effusions was limited. Nonetheless, our study shows that soluble apoptotic markers may be meaningfully associated with malignant pleural effusions, particularly in conjunction with the fibrinolytic system. Further research is required to validate our results in larger randomized prospective trials.

CONCLUSION

This study demonstrates that Fas and TRAIL levels and P/B ratios of fibrinolytic factors are significantly elevated in patients with malignant pleural effusion. Elevated Fas levels were found to be closely linked to the prevalence of malignant effusion. These results suggest that measuring the Fas and TRAIL levels and calculating the P/B ratio of fibrinolytic factors may offer additional benefits in screening patients with suspected malignant pleural effusion.

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Author contributions: JW Choi designed the study, organized the research and wrote the manuscript. MH Lee analyzed the data, prepared the tables and reviewed the drafts of the manuscript. T Fujii performed the statistical analyses, performed literature searches, and edited the manuscript.

Conflict of interest disclosure: The authors have no conflict of interest to declare.

Data availability: The data used to support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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