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Combining ADA, Protein and IFN-γ Best Allows Discrimination Between Tuberculous and Malignant Pleural Effusion

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Background : *The purpose of this study is to assess the usefulness of various enzymes, cytokines and biochemical studies of pleural fluid for the differential diagnosis of tuberculosis from malignant pleural effusions, and to clarify the role of combining diagnostic tests.*

Methods : *The study group included 39 cases with tuberculous effusions and 31 cases with malignant effusions, whose diagnoses were confirmed by pleural biopsy, cytology or microbiological methods. We compared pleural fluid levels of ADA, TNF-α, IFN-γ, IL-2, IL-6, IL-8, pH, protein, glucose, cholesterol, triglyceride, amylase and lactic dehydrogenase between tuberculous and malignant effusions. Using stepwise logistic regression analysis, we evaluated the benefit of combining various parameters. Receiver operating characteristic(ROC) curves of ADA, cytokines and equations generated from regression analyses were plotted and compared with the area under curve(AUC). Cut-off values showing the best diagnostic accuracy were selected and compared.*

Results : *Compared to malignant effusion, tuberculous effusion showed significantly higher levels of ADA, IFN-γ, TNF-α and IL-2. There was a good correlation between IFN-γ and TNF-α. By stepwise logistic regression analysis, IFN-γ, protein and ADA were independent variables predicting tuberculous from malignant effusions. The diagnostic accuracy and AUC of regression equation was greater than any other single parameters.*

Conclusion : *For the differential diagnosis of tuberculosis and malignant pleural effusions, combining ADA, protein and IFN-γ best allows discrimination.*

Key Words : *Pleural effusion, Tuberculosis, Cytokine, ROC curve*

INTRODUCTION

Although the incidence of tuberculous pleuritis is the highest among the exudative pleural effusions in Korea[1], the differentiation of tuberculosis from malignant pleural effusion remains elusive in many cases even with the aid of biochemical, microbiologic and pathological analyses.

Measuring adenosine deaminase(ADA) from the pleural fluid is a useful diagnostic test, since an elevated level of pleural fluid ADA is a sensitive and specific indicator of tuberculous pleuritis[2-5]. As a delayed hypersensitivity appears to play a large role in the pathogenesis of tuberculous pleuritis[6-7], diagnostic significances of a number of cytokines, including interferon-γ (IFN-γ)[5, 8-11], tumor necrosis factor-α (TNF-α)[10, 11], interleukin 2 (IL-2)[9, 12] and interleukin 6 (IL-6)[13] have been studied.

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Interleukin 8 (IL-8) is induced by lipoarabinomannan of tuberculous bacilli[14], thus it is also expected to be concentrated in tuberculous effusion.

However, in consideration of clinical application of these parameters, it is required to compare their diagnostic significance and to clarify whether there is an additive benefit of combining diagnostic parameters. In a previous study by the authors[15], we found that combining TNF-α had no further diagnostic benefit than ADA alone, despite significant difference of TNF-α and ADA between tuberculous and malignant pleural effusion. In this study, we also measured IFN-γ, IL-2, 6 and 8 with the same samples as our previous study[15] and evaluated diagnostic significance of the markers and various combinations. Our result shows that IFN-γ, protein and ADA best allow discrimination of tuberculous effusion from malignant effusion.

MATERIALS AND METHODS

1. Subjects

Seventy patients (49 males and 21 females) referred to our hospital from January 1993 to July 1995 for the diagnosis of exudative pleural effusion and subsequently confirmed to have tuberculous (n=39) and malignant (n=31) effusions were studied. Tuberculous pleuritis was diagnosed either by detection of acid fast bacilli or culture of *Mycobacterium tuberculosis* from the pleural fluid or by the histology of a pleural biopsy specimen. The diagnoses of malignant effusion were made by cytopathologic detection of malignant cells in the effusion or pleural biopsy specimens. Those whose definitive etiology was not determined with the methods described above were excluded from this study.

2. Methods

Aliquots of pleural fluid obtained by thoracentesis were examined for differential cell counts, bacterial and mycobacterial stain and cytology. Samples were processed for bacteriologic culture and for the determination of pH, protein, glucose, cholesterol, triglyceride, amylase and lactic dehydrogenase(LDH). About 20ml of pleural fluid was centrifuged at 2,500rpm for 10 min to pellet the cellular element, and the supernatants were stored at -70℃. Pleural biopsy was performed with Cope biopsy needle.

ADA activity assay : The ADA activity was measured with a commercial assay kit (Toyobo Co., Osaka, Japan) along with a routine diagnostic work-up. By the catalytic reaction of ADA, inosine is generated, which is converted to uric acid and hydrogen peroxide. With an addition of peroxidase, absorbance was measured at 546 nm. The ADA activity could be measured in 47 of 70 subjects because of scheduling.

Cytokines assay : Deep frozen pleural fluid samples were melted at room temperature and transferred to assay tubes for radioimmunoassay (Advanced Magnetics Inc, Cambridge, MA) of TNF-α, IFN-γ, IL-2, IL-6 and IL-8. Each measurement was duplicated and expressed as the mean of two values. The principle of assay is based upon competition of cytokines in pleural fluid with radioactively labeled cytokines for a limited number of sites on a specific antibody. The higher the concentration of the cytokines in the pleural fluids, the less labeled cytokines are bound to the antibody. Antibody-bound cytokines are separated from unbound cytokines with magnetic goat anti-rabbit Ig-G through magnetic separation or centrifugation. The antibody-bound labeled cytokines are quantified by counting in a gamma counter(COBRA 5003, Hewlett-Packard, Meriden, CT). The counting rate is correlated with concentration of a standard curve. We measured the cytokines in two separate sessions. Firstly, all of IFN-γ, TNF-α, IL-2, IL-6 and IL-8 were measured for 36 samples. We measured only IFN-γ and TNF-α for the remaining 34 cases because of lack of diagnostic usefulness of interleukins according to the preliminary assessment.

3. Statistical analysis

Group data are shown as the means±standard deviations(SD). The differences among groups were tested by means of Student's t-test for parametric variables or Mann-Whitney U test for non-parametric variables. We used a stepwise logistic regression analysis to estimate the contribution of various parameters for the differential diagnosis. The p value allowing variables to be

entered in the regression equation was set to 0.05. ROC curves were plotted with varying degree of cut-off values for ADA, protein, cytokines and equations generated from regression analyses. To compare their diagnostic significance, the area under the ROC curves was calculated and compared with the methods reported previously[16, 17]. The best cut-off values for the parameters and regression equations were marked for values showing highest accuracy. We used BMDP statistical software(BMDP statistical software Inc, Los Angeles, CA). The statistical significance was defined as two tailed p value less than 0.05.

RESULTS

The characteristics of the subjects are listed in Table 1. The amount of smoking was significantly higher in subjects with malignant effusions ($p < 0.05$). Biochemical and cellular analysis results were not different between the two groups (Table 2). Of 67 cases whose protein contents were determined, 22 cases(32.8%) showed protein contents above 5g/dL. Among the 22 cases with protein contents higher than 5g/dL, 17(77.3%) were tuberculous, while only 5(22.7%) were malignant effusion. In contrast, of 45 cases with protein contents below 5g/dL, 21 cases were tuberculosis and 24 cases were malignant effusion(Chi square : 4.46, $p<0.05$). ADA, IFN-γ, TNF-α and IL-2 were significantly higher in tuberculous effusion than malignant effusion, but IL-6 and IL-8 were not different(Table 3).

There were good correlations between IFN-γ, TNF-α and ADA, while no significant correlations were noted between cytokines and protein (Table 4). For the stepwise logistic regression analysis, ADA, IFN-γ, TNF-α, IL-2, IL-6, IL-8, total protein, lymphocyte proportion, glucose and pH were submitted initially as independent variables. After eliminating variables with little relationship to the differential diagnosis, ADA, IFN-γ, TNF-α and total protein were submitted to the regression

Table 1. Characteristics of Subjects According to the Etiology of Pleural Effusion

	Tuberculosis Effusion	Malignant Effusion
Number	39	31
Age (years)	54.6±16.2	58.6±12.5
Sex (M/F)	27 / 12	22 / 9
Amount Smoked (pack.years)	24.0±10.0	38.6±21.9*

*: $p<0.05$ Data was expressed as mean±standard deviation.

Table 2. Comparisons of Biochemical Parameters Between Tuberculous and Malignant Pleural Effusions

	Tuberculosis(39)	Malignant Effusion(31)
WBC (/μL)	3536±3301 (38)	4072±8261 (28)
Neutrophil (%)	48.2±30.9 (38)	56.1±27.9 (27)
Lymphocyte(%)	51.2±30.2 (38)	43.9±27.9 (27)
Glucose (mg/dL)	101.1±55.8 (38)	111.3±59.9 (29)
Protein (g/dL)	4.57±1.37 (38)	4.05±1.0 (29)
Amylase (U/L)	47.7±20.3 (27)	51.6±24.4 (21)
LDH# (U/L)	964.9±810.8(35)	1197.9±1569.5(28)
Triglyceride (mg/dL)	36.8±30.7 (29)	26.9±14.1 (20)
Cholesterol (mg/dL)	98.3±62.4 (29)	86.8±37.5 (20)
pH	7.41±0.37 (37)	7.36±0.12 (27)

LDH : lactate dehydrogenase
Values in parentheses are number of subjects compared. Data was expressed as mean±standard deviation, and none of the variables showed significant differences between the two groups.

Table 3. Comparisons of Pleural Fluid Adenosine Deaminase(ADA) and Cytokines Between Tuberculous and Malignant Pleural Effusions

	Tuberculosis Effusion(39)	Malignant Effusion(31)
ADA(IU/L) (55)	48.7± 32.7(27)	13.9±13.0(20)***
IFN-γ (IU/mL)(79)	38.7± 43.2(39)	1.8±5.7(31)***
TNF-α (pg/mL)(79)	184.1± 214.2(39)	41.4±124.0(31)**
IL-2(ng/mL)(40)	134.6± 99.8(21)	85.3±12.5(15)*
IL-6(pg/0.1mL)(40)	351.5±164.1(21)	262.5±125.7(15)
IL-8(pg/0.1mL)(40)	284.7±744.0(21)	356.4±1236.1(15)

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$
Data was expressed as mean±standard deviation.
Values in parentheses are number of subjects compared.
Abbreviations : IFN-γ : Interferon-gamma,
TNF-α : Tumor necrosis factor alpha,
IL-2, 6, 8 : Interleukins 2, 6 and 8

analysis. With 47 subjects of tuberculous and malignant effusions, ADA was firstly entered to the equation followed by protein and IFN-γ (Regression Equation, RE=0.056×ADA+1.102×Protein+0.193×IFN-γ-6.829, 0 : malignant, 1 : tuberculosis, Table 5).

Table 4. Correlations Between ADA, TNF-α, IFN-γ, IL-2, IL-6 and Protein

	ADA (47)	IFN-γ (70)	TNF-α (70)	IL-2 (36)	IL-6 (36)	IL-8 (36)
IFN-γ (70)	0.55*** (47)					
TNF-α (70)	0.34** (47)	0.78*** (70)				
IL-2 (36)	0.02 (25)	0.07 (36)	0.08 (36)			
IL-6 (36)	0.52** (25)	0.37* (36)	0.35* (36)	0.13 (36)		
IL-8 (36)	0.15 (25)	-0.12 (36)	-0.14 (36)	0.49** (36)	0.42** (36)	
Protein (67)	0.06 (47)	0.06 (67)	0.01 (67)	0.18 (35)	0.08 (35)	-0.15 (35)

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$
Values in parentheses are number of subjects compared. Abbreviations : see Table 3

The AUC of regression equation was 0.92 which was greater than those of any other single parameters(Fig. 1). According to the cut-off values showing the best diagnostic accuracy, the diagnostic significance of various parameters and regression equations was compared(Table 6). The diagnostic accuracy of regression equation was the best among the other parameters.

DISCUSSION

For the differential diagnosis of tuberculous and malignant pleural effusions, commonly used diagnostic tests, including protein, LDH, glucose, pH and lymphocyte proportions, were reported not to be useful[18]. Our findings are consistent with earlier studies except protein. Although the mean values of protein concentrations of tuberculous and malignant pleural effusions were not different, tuberculous pleural effusion was significantly prevalent among those who showed protein content higher than 5.0g/dL. Of 22 cases whose pleural fluid

Table 5. Results of Stepwise Logistic Regression Analysis and Generated Regression Equations for the Differential Diagnosis of Tuberculous and Malignant Effusions

	Coefficient	Odd ratio	95% Confidence Interval	Significance
ADA	0.056	1.06	1.01 -1.11	$p<0.001$
Protein	1.102	3.01	0.986-9.18	p=0.006
IFN-γ	0.193	1.21	0.939-1.57	p=0.005
(Constant)	- 6.829	0.001		

Regression Equation(0 : malignant, 1 : tuberculosis)=0.056×ADA+1.102×Protein+0.193×IFN-γ-6.829
Abbreviations : see Table 3

Table 6. Comparison of Sensitivity, Specificity and Accuracy for the Differential Diagnosis of Tuberculous and Malignant Effusions

	Cut-off value*	Sensitivity	Specificity	Accuracy
ADA (IU/L)	32	66.7	90.0	76.6
IFN-γ (IU/mL)	9.1	74.4	93.5	82.9
TNF-α (pg/mL)	117	69.2	87.1	77.1
IL-2 (ng/mL)	97	66.7	86.7	75.0
IL-6 (pg/0.1mL)	360	61.9	80.0	69.4
Protein (g/dL)	5.3	50.0	82.8	64.2
RE#	0.6	81.5	95.0	87.2

*: The cut-off values showing the best accuracy for the parameters selected.
Abbreviations : see Table 3. #RE : regression equation(see Table 5)

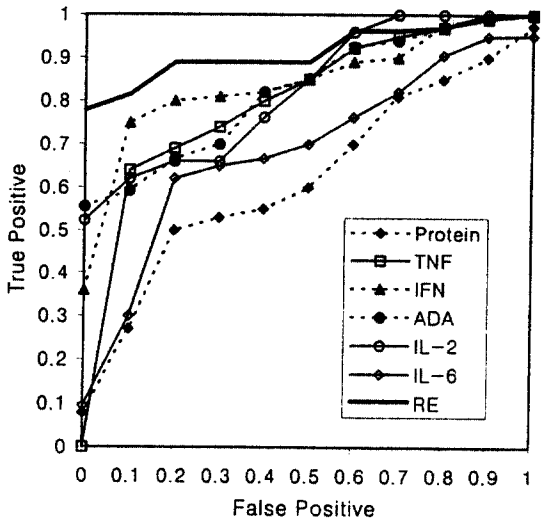

Fig. 1. Receiver operating characteristics (ROC) curves for IFN-γ, TNF-α, ADA, IL-2, IL-6, Protein, and regression equation for the differential diagnosis of tuberculous from malignant effusion. Area under curve (AUC) : IFN-γ (0.84), TNF-α (0.81), ADA (0.84), IL-2 (0.83), IL-6 (0.69), Protein(0.62), RE (0.92) Abbreviations : see Table 3., RE : regression equation (see Table 5).

protein contents were higher than 5g/dL, 17(77.3%) were tuberculous. Light et al.[19] also reported that in many cases with tuberculous pleural effusion, protein contents were high, frequently above 5.0g/dL.

Adenosine deaminase is an enzyme of purine catabolism which catalyzes the pathway from adenosine to inosine and is found predominantly in T lymphocytes. In an investigation[2], the pleural fluid ADA above 70IU/L indicated a high probability of tuberculosis, whereas ADA below 40IU/L indicated very low probability of tuberculosis. This finding was also observed by a number of studies[3-5]. In this study, ADA was significantly different between the two groups and the diagnostic accuracy reached maximal value with a cut-off value of 32IU/L.

IFN-γ enhances hydrogen peroxide production by macrophages[20] and may facilitate intracellular elimination of mycobacteria[21]. Similarly, TNF increases the phagocytic capacity of macrophages and enhances mycobacterial killing by human macrophages in vitro[22, 23]. The local production of TNF and IFN-γ in tuberculous pleural fluid was also demonstrated[10]. Increased IFN-γ in tuberculous effusion was reported by a number of studies[5, 8-11]. Ribera et al.[8] noted that the mean level of IFN-γ in all tuberculous pleural fluid had levels above 2.3U/mL. In contrast, the IFN-γ levels were below 2.0U/mL in non-tuberculous pleural effusions. TNF-α, however, was not reported to be significantly different between tuberculous and non-tuberculous pleural effusions[11]. Our result was different from them; both IFN-γ and TNF-α were significantly elevated in tuberculous fluids and they showed a good correlation with each other.

The significance of IL-2 and IL-6 in differentiation of tuberculous and malignant pleural effusions was also studied by several investigators[9, 12, 13]. Although they were reported to be concentrated in tuberculous pleural effusions, we noted significant differences in IL-2 but not in IL-6 in the present data. While IL-8 was also expected to be elevated in tuberculous effusion, as it is induced by lipoarabinomannan of tuberculous bacilli[14], there was no significant difference between tuberculous and malignant effusion in the present result.

In this study, we evaluated the combination of several diagnostic test results for the differentiation of malignant and tuberculous effusions. There were several studies which evaluated the diagnostic significance of multiple parameters concurrently[4, 5, 9]. Shimokata et al.[9] studied IL-1β, IL-2, IFN-γ and ADA in malignant and tuberculous pleurisies. However, they did not compare the diagnostic value of each of the parameters. De Oliveira et al.[4] reported the high diagnostic significance of combination of ADA and lymphocyte proportion, which we could not confirm in this study because both tuberculous and malignant effusions showed high lymphocyte proportions. Valdes et al.[5] compared serum and pleural fluid ADA, lysozyme and IFN-γ, and concluded that none of the other parameters but IFN-γ and ADA was useful. Although they did not show whether both of the parameters were needed for the differentiation of exudative pleural effusions, their findings are consistent with the present data.

In an attempt to clarify the benefit of combining diagnostic tests, we evaluated the diagnostic sig-

nificance of combining various parameters with stepwise logistic regression analyses. For the differential diagnosis of tuberculous and malignant pleural effusions, we found that not only ADA and IFN-γ but also protein were independent variables.

It is of interest that protein is one of the independent discriminators of tuberculosis from malignant effusions. Although the difference of protein contents was not significant between the two groups, the probability of tuberculosis was significantly higher in patients who showed pleural fluid protein content higher than 5.0g/dL. As there was no correlation between ADA, IFN-γ and protein, adding protein might have detected additional tuberculous pleuritis with relatively low ADA or IFN-γ contents. Thus, although protein itself did not show good diagnostic accuracy, it showed a benefit by adding on ADA. We could further confirm the diagnostic benefit of combining ADA, protein and IFN-γ with a comparison of diagnostic accuracy and the AUC of ROC curves.

While TNF-α showed a significant difference between tuberculous and malignant pleural effusion, it was not entered to the regression equations after the effect of other parameters. This can be explained with the excellent correlation between IFN-γ and TNF-α. Hence, if either of IFN-γ or TNF-α is used already, adding the other one does not enhance the diagnostic accuracy.

From these results we conclude that combining ADA, protein and IFN-γ best allows discrimination between tuberculosis and malignant pleural effusion.

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