

ANTI-ENDOTHELIAL CELL ANTIBODIES IN PATIENTS WITH INTERSTITIAL LUNG DISEASES

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Title page

**ANTI-ENDOTHELIAL CELL ANTIBODIES IN PATIENTS WITH INTERSTITIAL
LUNG DISEASES**

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Running head: AECA in Interstitial Lung Diseases

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Abstract

Background: Anti-endothelial cell antibodies (AECA) are circulating antibodies that bind to endothelial antigens and induce endothelial cell damage. AECA have been detected in patients with collagen vascular disease (CVD) and their presence is associated with interstitial lung disease (ILD) in cases of CVD. However, the prevalence of AECA in patients with idiopathic interstitial pneumonias (IIPs) is not known.

Methods: We investigated the prevalence of AECA in patients with IIPs. We also examined whether the expression of AECA differed among the histologic subgroups usual interstitial pneumonia (UIP) and nonspecific interstitial pneumonia (NSIP), and compared the values with those of CVD-associated ILD (CVD-ILD). Twenty patients with IIPs and 24 patients with CVD-ILD were studied. Serum samples were examined for AECA by cellular enzyme-linked immunosorbent assay (ELISA) using human umbilical vein endothelial cells. Values are expressed as ELISA ratios (ER).

Results: All sera from patients with IPF/UIP were negative for AECA, whereas 5 out of 10 with idiopathic NSIP, 5 out of 14 with CVD-UIP, and 4 out of 10 with CVD-NSIP tested positive ($p < 0.05$). ER values were significantly lower in patients with IPF/UIP than

idiopathic NSIP, CVD-UIP or CVD-NSIP ($p < 0.05$). Among idiopathic NSIP, CVD-NSIP and CVD-UIP patients, the ER values did not differ.

Conclusions: Among IIP patients, only those with idiopathic NSIP, not IPF/UIP, tested positive for AECA. The prevalence of AECA in idiopathic NSIP patients was similar to that in CVD-ILD patients. These results may provide important information to understand the distinct pathophysiology of each form of IIPs.

Text

INTRODUCTION

Anti-endothelial cell antibodies (AECA) are a heterogeneous group of antibodies recognizing various antigenic determinants on human endothelial cells. Although their precise pathogenic role remains unclear, studies *in vitro* suggest that AECA bind to endothelial cell membrane antigens and damage endothelial cell either through antibody-dependent cell-mediated cytotoxicity or the recruitment of natural killer cells [1-3]. AECA also upregulate the expression of adhesion molecules on endothelial cells and induce the secretion of cytokines and chemokines, which cause leukocyte homing, recruitment and adhesion to endothelial cells [4,5]. AECA have been detected in patients with collagen vascular diseases (CVDs), including systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and rheumatoid arthritis (RA), as well as in patients with systemic vasculitic diseases [6-13]. Although the prevalence of AECA varies among these diseases, it is correlated with disease activity and the degree of organ damage, and is regarded as a risk factor for disease relapse in vasculitis [7-12,16].

Interstitial lung diseases (ILDs) are a group of diseases that involve the space between the epithelial and endothelial basement membranes. ILDs have hundreds of etiological factors,

including environmental exposure and drugs. ILDs are common in patients with CVD and are a major determinant of their prognosis. Recent studies have shown a relationship between AECA and ILD in patients with CVD. In polymyositis and dermatomyositis (PM/DM), AECA were detected significantly more frequently in patients with PM/DM associated ILD than in those with PM/DM only [14, 15]. In patients with SSc, AECA were found to be associated with ILD and an indicator of alveolar-capillary impairment [16].

Idiopathic interstitial pneumonias (IIPs) are a category of diseases of unknown etiology and are recommended to be further subdivided into clinico-radiologic-pathologic entities [17]. Among IIPs, idiopathic pulmonary fibrosis (IPF), which has the pathological features of usual interstitial pneumonia (UIP), and nonspecific interstitial pneumonia (NSIP), are perhaps the most clinically important and frequently occurring. It is critical to differentiate between IPF/UIP and NSIP pathologically, because of differences in survival and response to corticosteroids.

Interestingly, endothelial cell injury has recently been shown to occur in a fibrotic process in patients with ILD, including those with IIPs and CVD-associated ILD (CVD-ILD) [18]. Thus, it is suggested that AECA are involved in endothelial cell injury in certain groups of IIPs because of a possible pathogenic role. To date, however, there has been no attempt to

examine the prevalence of AECA in patients with IIPs. In the present study, we aimed to evaluate the prevalence of AECA directed against human umbilical vein endothelial cells (HUVEC) in patients with IIPs. In addition, we examined whether the prevalence and levels of AECA differ among the histologic subgroups of IIPs, and compared the values with those for CVD-ILD. Finally, we further studied the relationship between AECA and clinical or serological features to clarify the clinical significance of these antibodies in patients with ILD.

MATERIALS AND METHODS

Patients and controls

Twenty patients with IIPs and 24 patients with CVD-ILD who consecutively underwent a surgical lung biopsy between 1998 and 2006 were included in this study. The diagnosis of IIP was based on clinical, radiographic, and pulmonary physiological features, according to the ATS/ERS consensus classification [17]. Histological classification of ILDs was performed based on pathologic findings in surgical lung biopsy specimens according to the current classification [17]. The underlying diseases in patients with CVD-ILD were RA (n = 6), SSc (n = 5), Sjögren syndrome (n = 6) and PM/DM (n = 7), all of which fulfilled the diagnostic criteria [19-22]. Serum samples were collected at the time of diagnosis, when none of the

patients were under systemic steroid or immunosuppressive therapy, and stored at -30°C until this investigation. Sera from 35 healthy volunteers matched for age and sex served as controls. The study protocol was approved by our institutional review board for human research. Informed consent was given by each patient.

Data collection

Clinical data, including sex, age, smoking history, symptoms and outcome was obtained from medical records. Pulmonary function tests and laboratory parameters, including serum lactate dehydrogenase (LDH), KL-6 and surfactant protein D (SP-D), were assessed.

ELISA for the detection of AECA

The ELISA to detect AECA in sera was performed as described previously (6), with some modifications. In brief, HUVEC at passage 3 were seeded in a 96-well flat-bottomed microtiter plate (Nunc, Denmark) at a concentration of 2×10^4 cells/well and allowed to grow to confluence as a monolayer for 2-3 days. Cells were fixed with 0.1% glutaraldehyde for 10 minutes at 4°C , and nonspecific binding sites were blocked with phosphate buffered saline (PBS)-bovine serum albumin (BSA, Sigma) for 1 h at 37°C . After 5 washes, 100 μl of serum

diluted 1:1000 in PBS-1% BSA was added to each well in triplicate for 1 h at 37°C. The wells were washed 5 times with PBS-1% BSA and incubated with horseradish peroxidase-conjugated rabbit F (ab')₂ anti-human IgG (DAKO, Denmark) diluted 1:800 in PBS-1% BSA for 1 h at 37°C. After 5 more washes with PBS-1% BSA, 3,3',5,5'-tetramethylbenzidine (TMB; DAKO, USA) was added as a substrate and incubated at room temperature. After 10 min, the enzyme reaction was stopped by the addition of 1M H₂SO₄, and optical density (OD) was measured at 450nm using an ELISA reader (BioTek, USA). The results were expressed as an ELISA ratio (ER) = (S-A) / (B-A), where S is the OD of the sample tested, and A and B indicate the OD of negative and positive controls, respectively. All assays included at least one positive and one negative control sample on each plate. As a positive control, a highly positive serum sample from an SLE patient without lung disease was used. As described in previous studies, a sample was judged to be positive when the ER was greater than the mean + 3 SD of the healthy control group.

Statistic analysis

All values were analyzed using StatView version 4.5 (Abacus Concept, CA). The Mann-Whitney U test and ANOVA were used for the statistical analysis. Correlations

between different parameters were evaluated with Spearman's rank correlation coefficient. P values less than 0.05 were considered significant. All data are expressed as means \pm SD.

RESULTS

Characteristics of patients

According to the histological findings, 10 of the IIP patients were classified as having UIP and 10 as having NSIP. In addition, among patients with NSIP, 2 patients were classified as having a cellular pattern and 8 patients were classified as having a fibrosing pattern. Using the same histological criteria, 14 of the CVD-ILD patients were categorized as having UIP and 10 as having NSIP. The clinical features and laboratory parameters are summarized in Table 1. Males predominated among those with IPF/UIP ($p < 0.05$). Age, smoking history and laboratory findings did not differ among the four groups. Serum levels of KL6 and SP-D, useful markers for the diagnosis and activity of interstitial pneumonia, also did not differ among the groups.

A control group comprising 21 males and 14 females with a mean age of 54 (range, 21 to 84) was studied to determine the cut-off value of AECA. These individuals did not show any clinical, radiological or serological evidence of pulmonary disease or CVD.

Expression of AECA in sera

In the control group, the ER value (mean \pm SD) of AECA was 0.246 ± 0.101 . There was no significant difference in the ER with regard to gender, age and smoking habits. The cut-off value of AECA was determined to be 0.549 using Rosenberg's method [6-8, 11, 15,16].

With this cut-off value, 5 of the 20 patients with IIP (25%) and 9 of the 24 patients with CVD-ILD (37.5%) were positive for AECA. Compared with control levels, the prevalence and ER values of AECA were significantly increased in both IIP patients and CVD-ILD patients. No significant difference was found in the ER between patients with IIPs and those with CVD-ILD (0.597 ± 0.259 and 0.476 ± 0.178 , respectively) (Figure 1). Next, we compared the expression of AECA among the histologic subgroups UIP and NSIP. Interestingly, all of the sera from patients with IPF/UIP tested negative for AECA, whereas the samples from 5 out of 10 patients with idiopathic NSIP (50%) were positive, indicating that the prevalence of AECA was significantly higher in cases of idiopathic NSIP than in cases of IPF/UIP. Among patients with idiopathic NSIP, one out of 2 patients with cellular NSIP and 4 out of 8 patients with fibrosing NSIP were positive for AECA. In CVD-ILD, 5 out of 14 patients with CVD-UIP (35.7%) and 4 out of 10 patients with CVD-NSIP (40%) were found to

be positive. As shown in Figure 2, the ER value of AECA was significantly lower in patients with IPF/UIP than those with idiopathic NSIP, CVD-UIP or CVD-NSIP ($p < 0.05$). Among patients with idiopathic NSIP, CVD-NSIP and CVD-UIP, the prevalence and ER values of AECA were not significantly different. In cases of CVD-ILD, the ER value and prevalence was not significantly different among the four underlying CVDs (data not shown).

Relationship between AECA levels and clinical parameters

The ER values of AECA in sera did not correlate with pulmonary function, laboratory parameters including serum levels of KL6, SP-D and other autoimmuno-antibodies, or the differential fraction of alveolar cells (Figure 3). Even among IIP patients only, ER values were not related to these findings. Of the IPF/UIP patients who tested negative for AECA, 5 died of respiratory failure. Among the idiopathic NSIP patients, one AECA-negative individual did not respond to corticosteroids and died of respiratory failure. Among the CVD-NSIP patients, one AECA-positive individual with SSc, who did not receive therapy because his respiratory condition was stable, died of renal crisis. Collectively, among our cases, there was no correlation between the presence of AECA and clinical features such as responsiveness to treatment and prognosis.

DISCUSSION

The present study investigated the expression and clinical significance of AECA in patients with IIPs compared with those with CVD-ILD. We found a significant difference in the prevalence and ER values of AECA among the histologic subgroups of IIPs. One half of all idiopathic NSIP patients had AECA, while none of the IPF/UIP patients did. In addition, the ER values of AECA were significantly higher in cases of idiopathic NSIP than in cases of IPF/UIP. By contrast, among patients with CVD-ILD, no difference was found in the prevalence or ER values of AECA, which were similar to those in cases of idiopathic NSIP, between patients with CVD-UIP and those with CVD-NSIP. There was no significant relationship between the expression of AECA and clinical or laboratory findings.

IIPs have been classified into subgroups, each with a distinct histologic pattern and prognosis. It has been suggested that each subgroup has a different pathologic process. In this context, it is interesting that AECA were only found in idiopathic NSIP patients (50%), and not IPF/UIP patients (0%). Although the precise role of AECA in ILD remains unclear, the variation in the expression of AECA among the histologic subgroups of IIPs may be associated with a distinct pathogenesis. Recently, Fujita et al. suggested that certain cases of idiopathic

NSIP could be considered identical to CVD-NSIP, because the clinical characteristics were quite similar despite the presence of underlying CVD [23]. Consistent with this, our previous study showed that in terms of prognosis and responsiveness to therapy, idiopathic NSIP was similar to CVD-NSIP [24]. Interestingly, the present study revealed the prevalence and ER values of AECA in patients with idiopathic NSIP to be comparable to those in patients with CVD-UIP and CVD-NSIP. In terms of the expression of AECA, idiopathic NSIP was more similar to CVD-ILD than IPF/UIP. As idiopathic NSIP is a provisional category that requires further elucidation and definition as described in the ATS/ERS statement [17], this antibody might be used to account for a part of this entity.

We evaluated the relationship between AECA expression and pulmonary function and laboratory findings, such as KL-6 levels and SP-D values. Impaired lung function, shown by a decreased FVC and low PaO₂ value in cases of ILD, is considered to reflect disease severity [25]. KL-6 is mainly expressed on type II pneumocytes and respiratory epithelial cells, and serves as a useful clinical marker that reflects the disease activity of ILD [26]. SP-D, a lung-specific protein, is also considered to be a useful marker of ILD [27]. In the present study, no relation was found between the ER values of AECA and these parameters. We also evaluated the relationship in patients with IIP only to exclude the influence of underlying CVD.

However, there was no significant correlation among these parameters. Thus, AECA cannot be used as a serum marker that represents disease activity or severity.

ILDs are characterized by damage to the lung parenchyma with varying patterns of inflammation and fibrosis, which mainly target alveolar epithelial cells [17,28,29]. In addition to these epithelial cell changes, it was recently reported that vascular damage is also present in patients with ILDs [18,30]. Takabatake et al. demonstrated pulmonary endothelial cell injury in ILD patients by assessing the kinetics of ^{123}I -metaiodobenzylguanidine (^{123}I -MIBG) [18]. In patients with ILDs, the rate at which ^{123}I -MIBG was washed out from the lungs, which reflects pulmonary endothelial cell injury, was reduced compared with that in normal controls. Moreover, the washout rate was correlated with parameters for disease severity. In Takabatake's study, however, it is not clear whether the degree of endothelial cell injury differed among histologic subgroups of ILD, because a pathologic diagnosis by surgical lung biopsy was not done. Based on the results of the present study, the expression of AECA may be attributable, in part, to the endothelial cell injury observed in ILD, especially idiopathic NSIP and CVD-ILD. On the other hand, a recent study reported that levels of vascular endothelial growth factor (VEGF) were decreased in BAL fluids in patients with IIPs and CVD-ILDs [30]. The reduction in VEGF, which plays a multifunctional role in the

maintenance of vascular structure and function, induces apoptosis of endothelial cells, leading to damage to the microcapillary endothelium. Taken together, these findings suggest that injury to the endothelial cells contributes in part to the pathological changes in patients with ILDs, and that AECA might be related to this process.

Recently, Margo et al indicated the presence of AECA in IPF patients [31]. In contrast to our study, twelve sera from 18 patients reacted with HUVEC. We suppose some reasons for this discrepancy. One is that they used an indirect immunofluorescence technique to detect AECA. Another reason is that the targeted patients differed greatly. They contained cases of pulmonary hemorrhage and lung transplantation. Moreover, all biopsies showed morphologic evidence of microvascular injury, which suggested ILDs associated with vasculitis rather than idiopathic ILDs. In this study, we included biopsy-proven IPF and idiopathic NSIP patients and excluded patients with apparent vasculitis. Studies of ILDs sometimes cause confusion, because intended patients differ from study to study. We need further studies in a larger cohort of biopsy-proven, selected idiopathic patients to improve our understanding of the role of AECA in IIPs.

In conclusion, among IIP patients, we detected AECA only in those with idiopathic NSIP, not those with IPF/UIP, and the prevalence and ER values of AECA in cases of idiopathic NSIP

were similar to those in cases of CVD-ILD. Although the role of AECA in ILD remains unclear, this difference in expression level between IPF/UIP patients and idiopathic NSIP patients might be associated with different pathologic processes. In addition, it is suggested that idiopathic NSIP is more similar to CVD-ILD than IPF/UIP in terms of AECA expression. These results may provide important knowledge that contributes to a better understanding of the distinct pathophysiology of each form of IIPs. Further studies will elucidate the characteristics of the antigen recognized by AECA and its precise role in the pathophysiology of ILDs.

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Table 1.

Patients' characteristics.

	IIP		CVD-associated ILD	
	IPF/UIP	Idiopathic NSIP	CVD-UIP	CVD-NSIP
Patients (<i>n</i>)	10	10	14	10
Age (years)	63.2±8.5	58.6±9.2	60.7±7.5	54.5±9.7
Male	10 (1 0 0) [‡]	3 (30)	10 (71)	4 (40)
Smoker	10 (1 0 0)	4 (40)	10 (71)	4 (40)
FVC (L)	2.7±0.8	2.3±0.7	2.3±0.8	2.2±0.8
FVC (%)	83.3±25.7	74.6±17.5	75.4±20.6	72.8±19.9
PaO ₂ (mmHg)	76.7±7.3	78.9±8.5	79.9±20.6	82.6±9.2
LDH (IU/L)	232±47	255±59	238±73	277±183
IgG (mg/dL)	1568±371	1555±306	1705±514	1845±460
KL-6 (U/mL)	936±727	2112±1699	1004±	

Data are presented as no or mean±SD.

[‡] $p < 0.05$ compared with idiopathic NSIP and CVD-NSIP.[†] $p < 0.05$ compared with IPF/UIP and CVD-UIP.

Figure legends

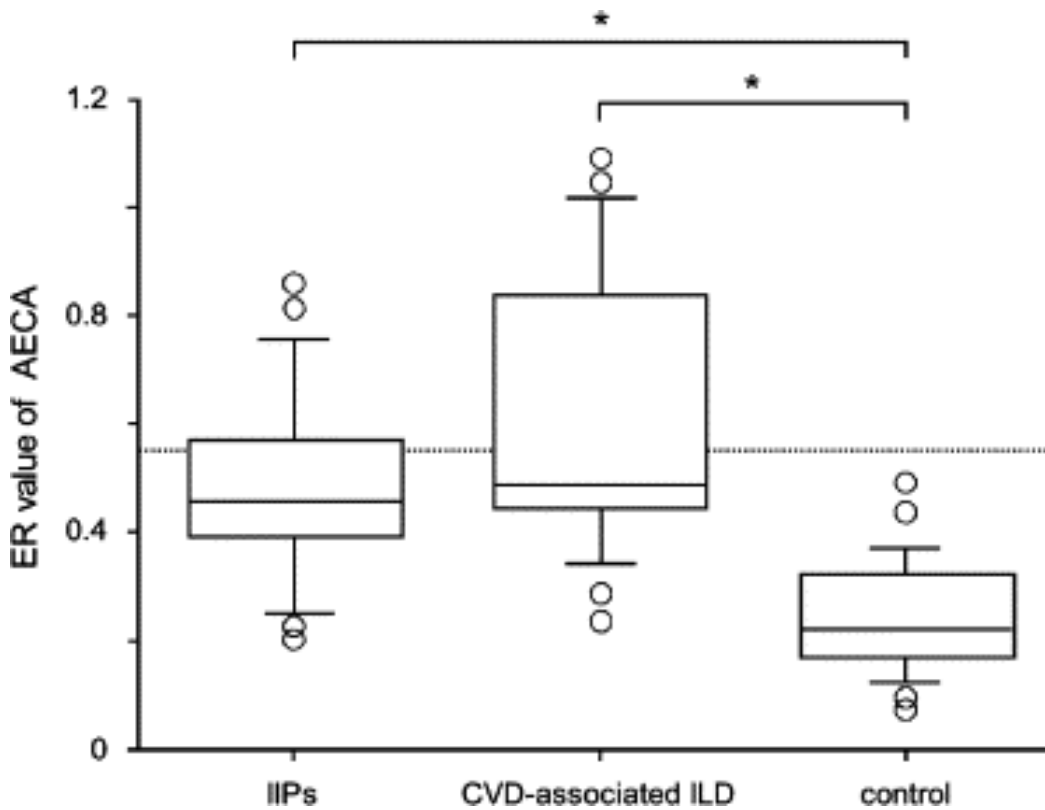


Figure 1. ER value of AECA in patients with IIPs, CVD-associated ILD and the control group.

The *dotted line* represents the cut-off value. *Horizontal lines, boxes, error bars* and *circles* represent the median, 25th and 75th percentile, 10th and 90th percentile, and data apart from 10th or 90th percentile, respectively.

Definition of abbreviations: AECA = Antiendothelial cell antibodies; ER = ELISA ratio.

* $p < 0.0001$

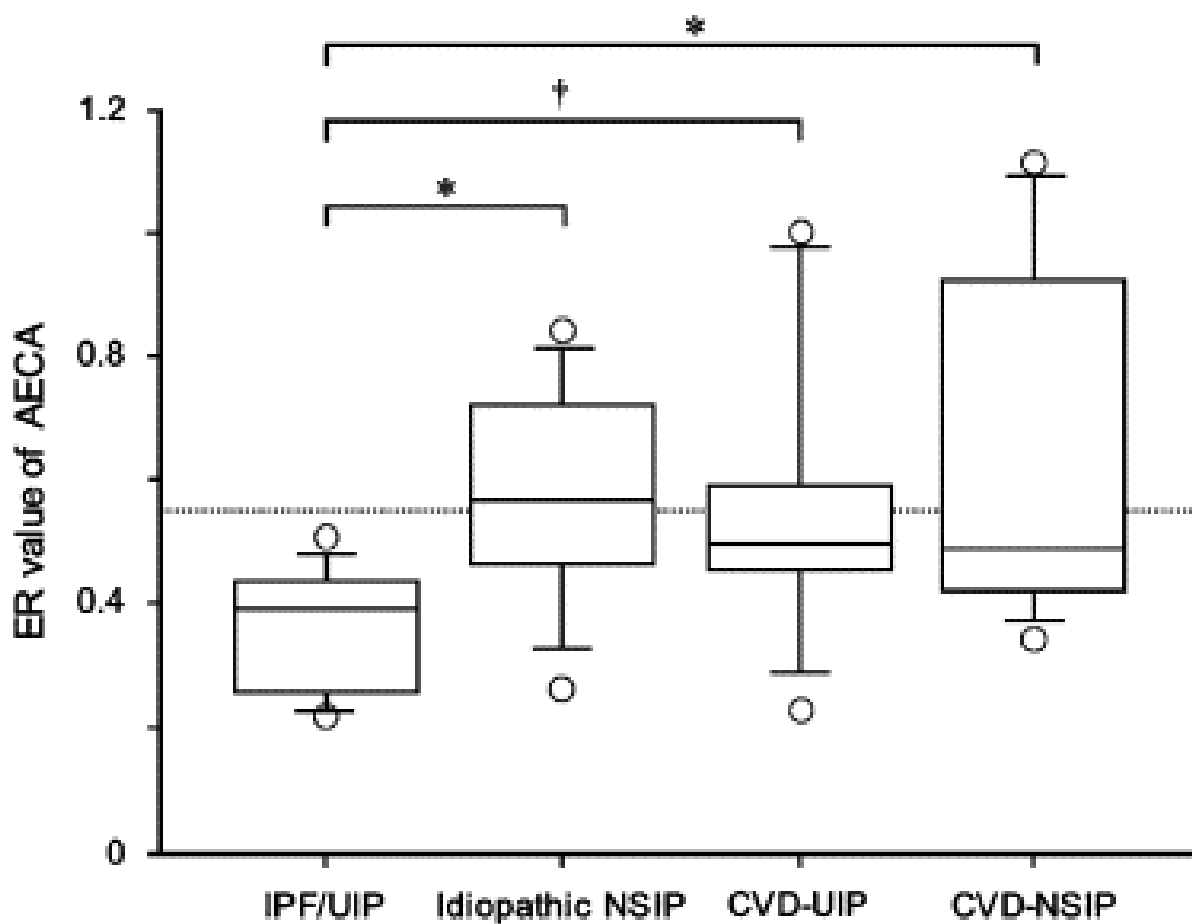


Figure 2. ER value of AECA in patients in each subgroup. The *dotted line* represents the cut-off value.

Specifics of the box plot are as described in Figure 1.

Definition of abbreviations: AECA = Antiendothelial cell antibodies; ER = ELISA ratio.

* $p < 0.01$, † $p < 0.05$

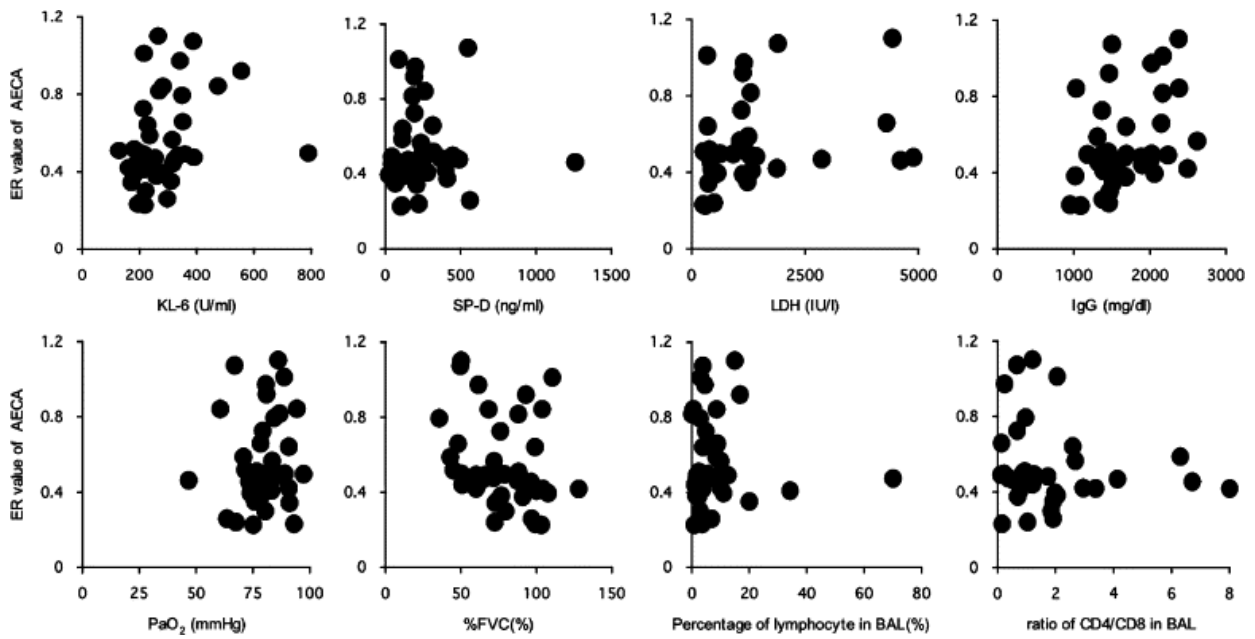


Figure 3. Association of AECA and the findings concerned with ILDs

Definition of abbreviations: AECA = Antiendothelial cell antibodies; ER = ELISA ratio;

BAL = bronchoalveolar lavage.