Surfactant protein D is associated with 3-month mortality of anti-MDA5 antibody-interstitial lung disease

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Abstract

Objective

To investigate the associations between serum levels of matrix metalloproteinase 7 (MMP7), surfactant protein D (SPD), interleukin 18 (IL-18) and chemokine ligand 18 (CCL18) with dermatomyositis and polymyositis-associated interstitial lung disease (DM/PM-ILD) and evaluate their prognostic values in the disease.

Methods

Seventy-eight patients with multiple disciplinary team diagnosis of DM/PM-ILD were enrolled and classified as anti-melanoma differentiation-associated protein 5 antibody (MDA5)-ILD, anti-synthetase antibodies (ARS)-ILD and other antibodies-ILD upon autoantibodies profiles. Clinical data were collected and serum levels of four biomarkers were analysed. The primary endpoint was 3-month mortality. The cut-off values of biomarkers for mortality were figured out by receiver operating characteristic (ROC) analysis. Cox regression was performed to evaluate predictive values.

Results

Serum levels of MMP7 (p=0.036), SPD (p<0.001), IL-18 (p<0.001) and CCL18 (p<0.001) in patients with DM/PM-ILD were significantly higher than healthy controls with levels of MMP7 (p=0.029) and SPD (p=0.029) in patients with MDA5-ILD significantly lower than patients with ARS-ILD. The 3-month mortality in MDA5-ILD was 54.5% (12/22). Multivariate analysis showed that age (p=0.001, HR 1.151, 95% CI 1.063–1.247) and an increased level of SPD (>75.90ng/ml, p=0.005, HR 16.411, 95% CI 2.369–113.711) were significant predictors for 3-month mortality in patients with MDA5-ILD.

Conclusion

Elevated serum biomarkers were associated with DM/PM-ILD with differential levels between MDA5-ILD and ARS-ILD. Age and an increased SPD had prognostic values for predicting short-term mortality in patients with MDA5-ILD. Our study was important in providing a clue for understanding the classification and prognosis of DM/PM-ILD.

Key words

dermatomyositis/polymyositis, interstitial lung disease, biomarkers, anti-MDA5 antibody, mortality

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Introduction

Idiopathic inflammatory myopathies (IIM) are a heterogeneous spectrum of disorders characterised by muscles weakness and inflammation affecting multiple organs such as the joints, skin and lungs (1, 2). The most common subgroups of IIM include dermatomyositis (DM), polymyositis (PM) inclusion body myositis (IBM) and immune-mediated necrotising myopathy (IMNM) (3). Interstitial lung disease (ILD) is the major complication of IIM, and is associated with an increased mortality in patients with DM and PM (4, 5). However, the pathogenesis of DM/PM-ILD is largely unknown.

The discoveries of myositis-specific autoantibodies (MSAs) have provided opportunities to understand the disease mechanisms. These autoantibodies are associated with distinctive clinical phenotypes. Among them, anti-aminoacyl-tRNA synthetase (ARS) antibodies and anti-melanoma differentiation-associated protein 5 (MDA5) antibody are strongly predictive of developing ILD. Anti-ARS antibodies-ILD (ARS-ILD), occurring in 80% of patients with anti-synthetase syndrome (ASS), is the leading cause of morbidity and mortality in patients with ASS (6). In particular, anti-MDA5 is associated with a very high incidence (74%) of rapidly progressive ILD with 6-month mortality rate of approximately 50% (7-9). But the potential causes leading to the different disease behaviour among autoantibody profiles remain uncertain. And the factors predicting short-term mortality in anti-MDA5-ILD (MDA5-ILD) have been rarely established.

Several studies reported that connective tissue disease-related ILDs (CTD-ILDs) shared similar clinical risk factors and serum biomarkers with idiopathic pulmonary fibrosis (IPF). For instance, higher levels of serum matrix metalloproteinase 7 (MMP7) (10), surfactant protein D (SPD) (11), interleukin 18 (IL-18) (12) and chemokine ligand 18 (CCL18) (13), had been identified to be related to disease severity and poor prognosis of IPF. Also, it had been demonstrated that peripheral blood levels of MMP7, SPD and CCL18 contributed to the early detection of rheumatoid arthritis-associated ILD (RA-ILD) (14). More recently, serum levels of IL-18 and CCL18 were reported to be negatively correlated with pulmonary functions in patients with systemic scleroderma-associated ILD (SSc-ILD) (15, 16). Based on these previous studies, we hypothesised that these serum biomarkers were associated with DM/PM-ILD with differential serum levels among autoantibody profiles. Furthermore, the predictive values of biomarkers for disease outcomes were of our interest.

In this study, we tested serological levels of MMP7, SPD, IL18 and CCL18 in patients with DM/PM-ILD and assessed their associations with the disease. Next, we investigated the comparative levels between patients with ARS-ILD and MDA5-ILD. And then, we evaluated whether the serum biomarkers had an impact on the 3-month mortality of MDA5-ILD.

Methods

Subjects

Patients with multiple disciplinary team (MDT) diagnosis of DM/PM-ILD in Nanjing University Medical School Affiliated Drum Tower Hospital from July 2018 to March 2019 were enrolled and all of them signed the written informed consent for clinical data and serum samples collection. DM/PM was diagnosed based on Bohan and Peter's criteria (17). Chest high-resolution computed tomography (HRCT) of all patients in supine position was performed at admission and assessed by a thoracic radiologist (X.X.). ILD was diagnosed when respiratory symptoms combined with radiographic abnormalities in HRCT findings according to the guidelines (18).

Baseline demographic information, clinical characteristics, radiographic findings and laboratory examinations including creatine kinase (CK), creatine kinase isoenzymes MB (CKMB), aspartate aminotransferase (AST), α-hydroxybutyrate dehydrogenase (α-HBDH), lactate dehydrogenase (LDH) and PaO_2/FiO_2 value were recorded. All patients were performed myositis-associated antibodies profiles detection, including anti-MDA5,

anti-Mi-2α, anti-Mi-2β, anti-TIF1γ, anti-NXP2, anti-SAE1, anti-Ku, anti-PM-Scl100, anti-PM-Scl75, anti-SRP, anti-RO-52 and anti-ARS (anti-Jo1, anti-PL7, anti-PL12, anti-EJ and anti-OJ). A subset of subjects had pulmonary function testing available, including forced vital capacity (FVC), FVC % predicted, diffusing capacity of the lung for carbon monoxide (DLCO) and DLCO % predicted.

The patients were classified as MDA5-ILD group and ARS-ILD group upon the presence of anti-ARS antibody or anti-MDA5 antibody. Patients with other myositis-specific autoantibodies were classified as other-ILD group. The primary endpoint was 3-month mortality. Age-and-gender-matched healthy controls were from the Physical Examination Center of Nanjing University Medical School Affiliated Drum Tower Hospital. This study was approved by the Ethics Committee of Nanjing University Medical School Affiliated Drum Tower Hospital according to the policy.

Detection of biomarkers

Vein blood samples in limosis were collected in admission before the usage of corticosteroids and immunosuppressive agents. Serum samples were separated by centrifuge at 3000g for 15 minutes. Sandwich ELISA was adopted to measure the serum levels of MMP7 (R&D Systems, USA), SPD (R&D Systems, USA), IL18 (RayBiotech, USA) and CCL18 (RayBiotech, USA) according to the manufacturers' instructions. Each sample had a duplicate.

Immunohistochemistry

Lung tissue samples were obtained from one patient with anti-MDA5 positivity by transbronchial cryobiopsy (TBCB). Lung samples had been fixed in 10% formalin and embedded in paraffin. First endogenous peroxidase activity was blocked with 0.3% H2O2 for 20 minutes. After being deparaffinised, the slides were preheated to 100°C for 20 minutes. Subsequently, the slides were incubated with the rabbit anti-human MMP7 (1:800, Bioss, China), SPD (1:1000, proteintech, USA), IL18 (1:1000, abcam, UK) and CCL18 (1:1600, Bioss, China) monoclonal antibodies for 1 h at 37°. Next, the sections were incubated with biotin-conjugated gout anti-rabbit IgG antibody (abcam, UK) for 15 minutes at 37°. Diaminobenzidine (DAB) chromogen solution was used for visualisation, and then the slides were counterstained with hematoxylin. Haematoxylin-eosin staining was also performed for the specimen.

Statistical analysis

All statistical analyses were performed by SPSS 22.0 and Graphpad Prism 7.0. One-sample Kolmogorov-Smirnov test was performed when needed. Mann-Whitney U-test was used to compare serum levels of four biomarkers (MMP7, SPD, IL18 and CCL18) between patients and healthy controls, and between patients with MDA-ILD and ARS-ILD. Receiver-operating characteristic (ROC) analysis was conducted to evaluate sensitivity and specificity of the associations of four biomarkers with DM/PM-ILD. Student's *t*-test was applied to compare continuous clinical variables including demographic features and laboratory results. Chi-square test was performed to compare categorical variables. Survival analysis was performed in MDA5-ILD group only. ROC was firstly performed to determine the cut-off values of serum levels of four biomarkers to create new binary variables. And then, cox regression model was used to assess the prognostic utility of four biomarkers for 3-month mortality. Kaplan-Meier curves and log-rank test was performed to evaluate the association between SPD levels and mortality. A *p*-value lower than 0.05 was considered to be of significance.

Results

Patient characteristics

The study included 78 patients with DM/PM-ILD with 16 (20.5%) patients reporting smoking history. They were 33 males and 45 females, with mean age of 55.62±11.53 years old (range: 32–86). Among them, there were 22 patients with anti-MDA5 positivity, whose mean age was 52.23±11.46 years old (range: 32–86), 44 of ARS-ILD group with mean age of 56.55±11.66 years old (range: 32–79) and 12 of other-ILD group, with mean age of 58.42±10.66 years old (range: 44–78).

As summarised in Table I, patients with MDA5-ILD were more likely to have fever (p=0.015) and rashes (p<0.001) compared to patients with ARS-ILD. At baseline, CK level (p=0.027) and PaO_2/FiO_2 (p=0.007) were significantly lower in patients with MDA5-ILD than those in ARS-ILD, while the values of AST (p=0.008) and LDH (p=0.041) were significantly higher in MDA5-ILD group than those in ARS-ILD group. The 3-month mortality was significantly higher (p<0.001) in patients with MDA5-ILD (12/22, 54.5%) compared to ARS-ILD (1/44, 2.3%). Clinical data of other-ILD group were not selected into statistical analysis due to the small sample size.

Serum levels of all biomarkers in patients with DM/PM-ILD

Serum levels of MMP7 (p=0.036), SPD (p<0.001), IL-18 (p<0.001) and CCL18 (p<0.001) were significantly increased in patients with DM/PM-ILD than those in healthy controls. When we compared serum levels of the biomarkers between MDA5-ILD group and ARS-ILD group, MMP7 (p=0.029) and SPD (p=0.029) were significantly lower in MDA5-ILD group compared to ARS-ILD group, while no significance was observed in IL18 and CCL18 levels between the two groups (Fig. 1). Receiver operating characteristic (ROC) analysis of the four biomarkers for DM/PM-ILD was assessed and the AUCs of each biomarker ranged from 0.614 to 0.860, with a combination reaching 0.940 (p<0.001; 95% CI 0.898–0.981) (Fig. 2).

Immunohistochemistry

The lung tissue was from a 60-year-old female, a non-survivor, with anti-MDA5 positivity. A pulmonary pathologist (F.M.) assessed the localisation and expression of the four biomarkers in lung tissues. The pathologic results showed that alveolar structures preserved with fibrin hyperplasia and fibrotic matrix located in pulmonary stroma. Alveolar septum widened with inflammatory cells infiltrating. Masson bodies inside the alveoli were also observed (Fig. 3a). MMP7 and SPD were

Table I. Baseline characteristics and differences among MDA5-ILD, ARS-ILD and other ILD.

Characteristic	MDA5-ILD n=22	ARS-ILD n=44	Other-ILD n=12	Healthy controls n=45
Age, mean ± SD	52.23 ± 11.46	56.55 ± 11.66	58.42 ± 10.66	53.82 ± 9.89
Male/Female, n	10/12	19/25	4/8	17/28
Smoking, n (%)	5 (22.7%)	10 (22.7%)	1 (8.3%)	
Fever*, n (%)	12 (54.5%)	12 (27.3%)	8 (66.7%)	
Rashes*, n (%)**	15 (68.2%)	10 (22.7%)	2 (16.7%)	
Joint diseases, n (%)	8 (36.4%)	11 (25%)	5 (41.7%)	
Muscle presence, n (%)	6 (27.3%)	11 (25%)	2 (16.7%)	
Reynaud phenomenon, n (%)	2 (9.1%)	1 (2.3%)	1 (8.3%)	
Mechanic's hands, n (%)	8 (36.4%)	9 (20.5%)	5 (41.7%)	
Crepitus, n (%)	18 (81.8%)	32 (72.7%)	10 (83.3%)	
CK*, mean ± SD	80.21 ± 87.17(n=19)	266.21 ± 518.50(n=43)	197.00 ± 409.12(n=12)	
CKMB, mean ± SD	19.63 ± 9.29(n=19)	18.70 ± 14.75(n=43)	29.42 ± 23.52(n=12)	
AST, mean ± SD**	66.84 ± 61.71(n=21)	26.39 ± 19.17(n=44)	45.475 ± 39.29(n=12)	
α-HBDH, mean ± SD	354.88 ± 235.80(n=17)	251.00 ± 120.05(n=43)	264.18 ± 106.61(n=11)	
LDH*, mean ± SD	579.86 ± 482.70(n=21)	344.50 ± 164.21(n=44)	378.58 ± 159.45(n=12)	
PaO_2/FiO_2, mean ± SD**	276.19 ± 112.06(n=20)	360.77 ± 111.61(n=41)	318.10 ± 110.97(n=11)	
3-month mortality*, n (%)**	12 (54.5%)	1 (2.3%)	1 (8.3%)	
FVC, mean ± SD	2.40 ± 0.46(n=6)	2.13 ± 0.74(n=22)	2.38 ± 1.43(n=7)	
FVC, % of predicted, mean ± SD	68.02 ± 16.95(n=6)	64.96 ± 19.62(n=21)	65.10 ± 25.29(n=7)	
DLCO, mean ± SD	4.59 ± 0.71(n=4)	3.76 ± 1.52(n=170	3.33 ± 1.16(n=5)	
DLCO, % of predicted, mean ± SD	56.70 ± 12.62(n=5)	47.00 ± 26.36(n=21)	56.95 ± 29.00(n=6)	

MDA5: anti-melanoma differentiation-associated protein 5 antibody; ARS: anti-amimoacyl-tRNA synthetase antibodies; ILD: interstitial lung disease; HC: healthy control; CK: creatine kinase; CKMB: creatine kinase isoenzymes MB-type: AST: aspartate aminotransferase; α-HBDH: hydroxybutyrate dehydrogenase; LDH: lactate dehydrogenase; PaO_2: partial pressure of arterial oxygen; FiO_2: fractional inspired oxygen concentration; FVC: forced vital capacity; DLCO: carbon monoxide diffusing capacity.
*Features of significant difference between MDA5-ILD group and ARS-ILD group.
*$p<0.05$, **$p<0.01$, ***$p<0.001$.

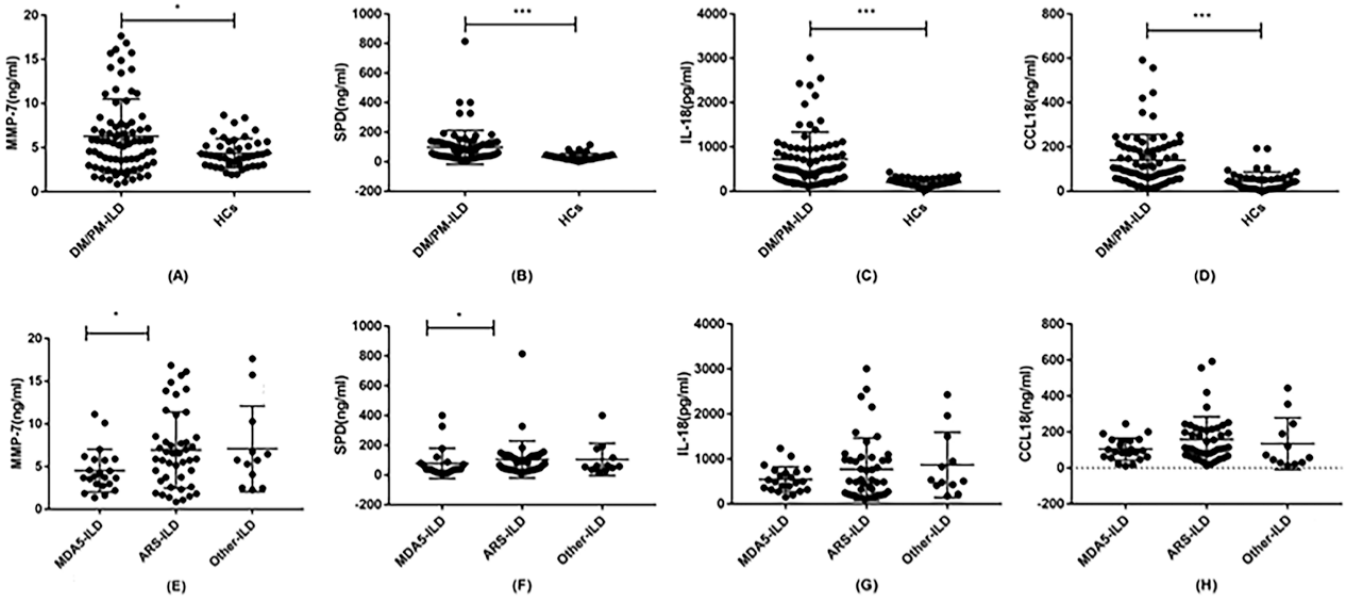

Fig. 1. Serum levels of four detected biomarkers in patients with DM/PM-ILD.
A-D: Serum levels of biomarkers between patients with DM/PM-ILD and healthy controls.
E-H: Serum levels of biomarkers among MDA-ILD, ARS-ILD and other-ILD.
*$p<0.05$, **$p<0.01$, ***$p<0.001$.

evidently expressed in type II alveolar epithelial cells (Fig. 3b-e). Meanwhile, IL18 and CCL18 were dominantly expressed in inflammatory cells, mainly lymphocytes, inside the pulmonary stroma (Fig. 3f-i).

Survival analysis

In this study, the mean follow-up time of all the patients with DM/PM-ILD was 9.96±4.42 (range: 1–15) months. In total, there were 14 non-survivors with a mean survival of 47.71±26.67 (range: 20–120) days from diagnosis. Twelve of them were with anti-MDA5 positivity, and two with anti-PL12 and antinuclear antibody, respectively. Survival analysis was performed only in MDA-ILD group because the majority

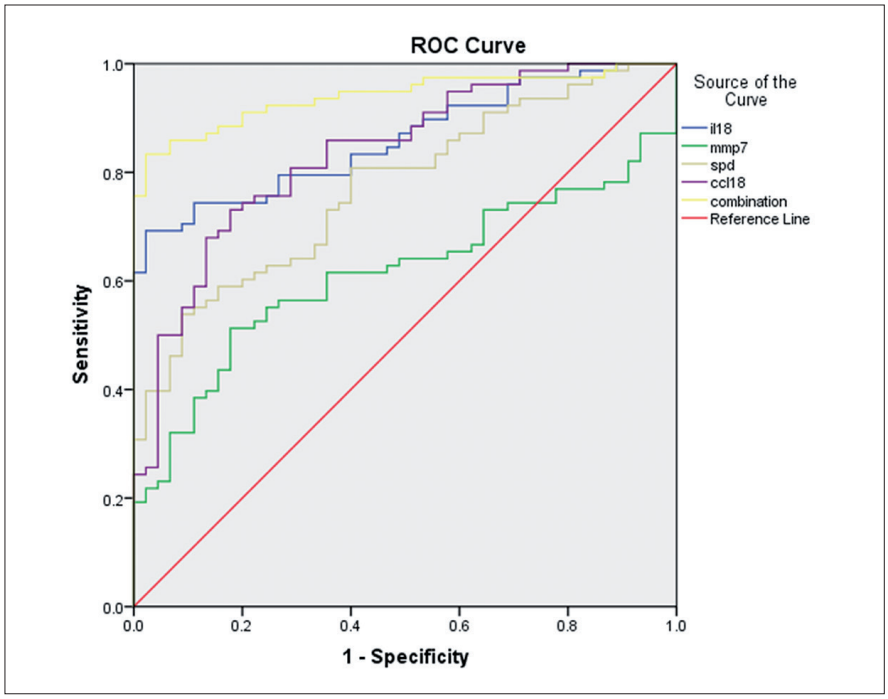

Fig. 2. ROC curves of biomarkers and the combination for DM/PM-ILD.
The AUCs of MMP7, SPD, IL18 and CCL18 curves were 0.614, 0.772, 0.860 and 0.835, respectively. The combination had an AUC reaching 0.940.

of non-survivors were with anti-MDA5 positivity. All the 12 non-surviors died from respiratory failure due to the progression of ILD and were all three-month mortality. Among the four biomarkers, serum levels of SPD was risk factor of 3-month mortality (p=0.013, HR 1.008, 95% CI 1.002–1.014) after adjustment for sex and smoking history. Then ROC curve of levels of four biomarkers provided cut-off values to conduct new binary variables. Univariate analysis by cox regression model showed that serum levels of SPD >75.90ng/ml (p=0.020, HR 4.076, 95% CI 1.244–13.360) was associated with worse survival. After adjustment for sex and smoking history by multivariate analysis, age (p=0.001, HR 1.151, 95% CI 1.063–1.247) and SPD >75.90mg/ml remained significant (p=0.005, HR 16.411, 95% CI 2.369–113.711) risk factors for predicting 3-month mortality in patients with MDA5-ILD. (Table II) Log-rank test also supported the above results. (p=0.013) (Fig. 4).

Discussion

The current study showed that serum levels of MMP7, SPD, IL18 and CCL18 elevated in patients with DM/PM-ILD, and a combination of four biomarkers had an enhanced association with the disease. The concentrations of SPD and MMP7 were significantly lower in patients with MDA5-ILD compared with those in ARS-ILD. Age and an increased level of SPD were significant risk factors for predicting 3-month mortality in patients with MDA5-ILD. This study revealed that four serum biomarkers were associated with DM/PM-ILD, offering clues to promote our understanding of the pathogenesis of the disease.

SPD is a member of the collectin family, which was previously demonstrated to take part in the innate immunity in lungs (19, 20). On the other hand, SPD was more commonly recognised as a biomarker of extracellular matrix (ECM) modifying in fibrotic progression as well as MMP7, a member of ma-

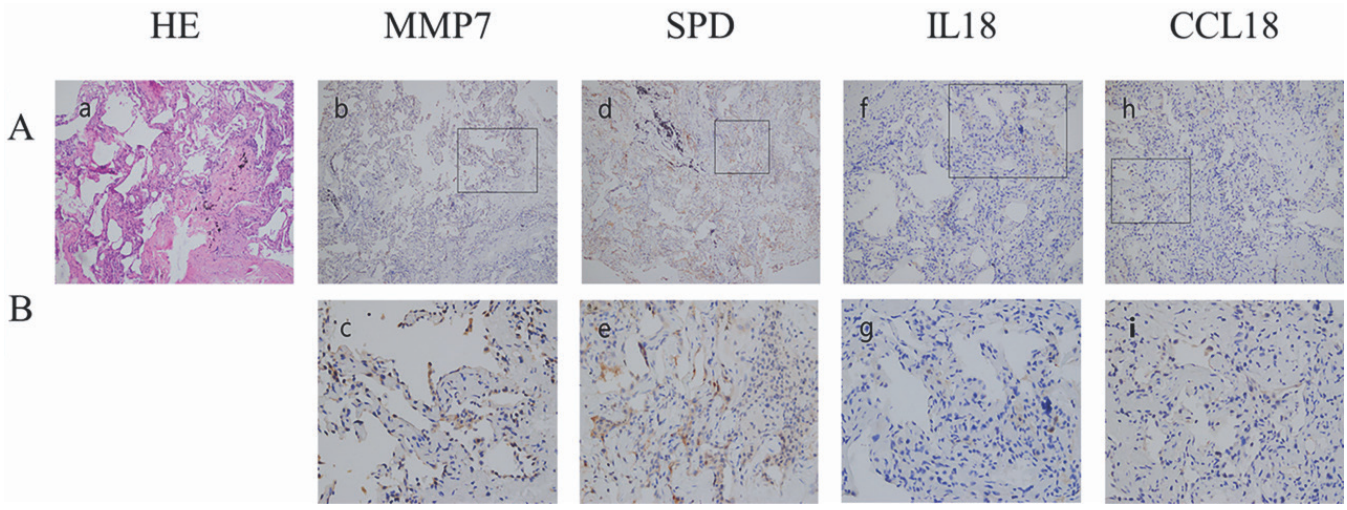

Fig. 3. Immunohistochemical results.
Pathologic results of an MDA5-ILD patient. **A**: Lower magnification, **B**: Higher maginification**.**
a: Haematoxylin and eosin (H&E) staining; **b-c**: staining with anti-MMP7 antibody; **d-e:** staining with anti-SPD antibody; **f-g:** staining with anti-IL18 antibody; **h-i:** staining with anti-CCL18 antibody.

Table II. Predictive factors for 3-month mortality in patients with MDA5-ILD.

Predictive factors	*p*-value	Hazard ratio (HR)	95% CI
Univariate			
MMP7>3.79ng/ml	0.183	2.279	0.678, 7.665
SPD>75.90ng/ml	**0.020**	**4.076**	**1.244, 13.360**
IL18> 342.95pg/ml	0.377	1.986	0.434, 9.094
CCL18>85.93ng/ml	0.470	1.560	0.467, 5.207
Multivariate			
SPD>75.90ng/ml	**0.005**	**16.411**	**2.369, 113.711**
Age	**0.001**	**1.151**	**1.063, 1.247**

MMP7: matrix metalloproteinase 7; SPD: surfactant protein D; IL18: interleukin 18; CCL18: chemokine ligand 18. The cut-off values of four biomarkers were obtained by receiver operating characteristic (ROC) analysis. The predictive values of each factor were assessed by cox regression model.

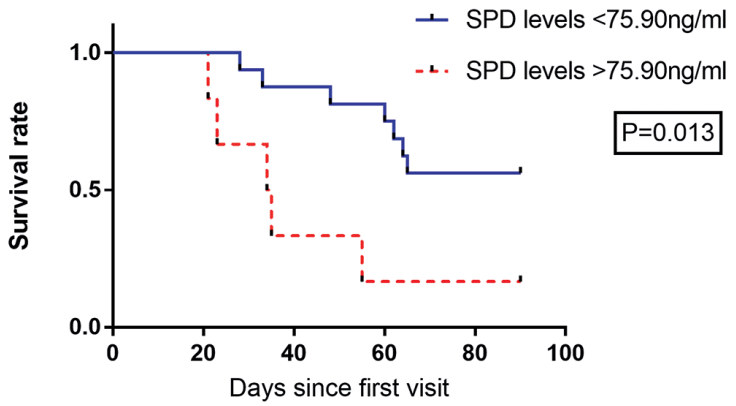

Fig. 4. Survival curves based on levels of serum SPD in MDA5-ILD group.
The cut-off value of SPD levels was determined by receiver operating characteristic (ROC) analysis. Log-rank test was performed to analyse the significance of two Kaplan-Meier curves. Time was represented in days.

trix metalloproteinases (MMPs) family, which increasingly expressed in injured lungs (21). These two lung-specific biomarkers were of interest in research of interstitial lung diseases especially IPF and CTD-ILDs for their defined functions in fibrotic process. It was noted that SPD and MMP7 might distinguish IPF from other ILDs by forming an index to enhance the diagnosis accuracy of IPF (22). Interestingly, SPD and MMP7 were also reported to be positively correlated with severity of fibrotic progression, resulting in worse pulmonary capacity in patients with interstitial lung diseases (10, 22). Similarly, our study demonstrated that the increased expressions of SPD and MMP7 were elevated in patients with DM/PM-ILD and dominantly located in type II alveolar epithelial cells, indicating that fibrotic process might play a role in the pathogenesis of the disease (23, 24).

IL-18 was secreted by activated macrophages and Kupffer cells, which induced IFN-γ production by TH1 cells and NK cells, was also expressed in airway epithelial cells to participate in T cell response (25, 26). This cytokine has been proved to be increasingly expressed in patients with IPF and sarcoidosis (27, 28). CCL18, secreted by antigen-presenting cells such as macrophages and dendritic cells, was chemotactic for T lymphocytes (29). High levels of CCL18 were associated with severity of inflammatory disorders such as Sjögren syndrome, rheumatoid arthritis and scleroderma (29). Previous mouse model study showed that CCL18 promoted collagen accumulation accompanied by T-lymphocytes infiltration (30). Our results of elevated serum IL18 and CCL18 levels reflected that macrophages activation and T cells chemotaxis, might participate in the occurrence of DM/PM-ILD.

The current classification, which include three most common subtypes (DM, PM and IBM) has some limitations because each criterion is not defined explicitly with some overlapping of clinical and histopathological features among these subgroups (2, 31). The discoveries of MSAs have provided new perspective to improve the disease classification (32), and distinctive phenotypes of specific autoantibodies have been widely reported (32). Consistent with previous study (8, 32), our results showed that patients with anti-MDA5 and anti-ARS had comparative clinical characteristics and significantly differential serum levels of biomarkers, suggesting the possibly distinctive pathogenesis of interstitial lung disease associated with different autoantibodies, which assisted in understanding classification of IIMs.

The short-term mortality of rapidly progressive ILD associated with anti-MDA5 was up to 50% (33). In current study, mortality rate in patients with MDA5-ILD (12/22, 54.5%) was significantly higher than those in non-MDA5 groups. Despite the high mortality of patients with MDA5-ILD, a cohort of 32 MDA5-ILD patients in China revealed that some patients did respond to treatment or manifested slowly progressive ILD, requiring efficient risk factors of the disease (34). Prognostic factors of worse survival in DM/PM-ILD including infections, skin ulcerations and decreased counts of lymphocyte have already been described (35, 36). But the predictive factors in patients with anti-MDA5 positivity were of lack. A study from Japan reported that elevated MMP7 levels were associated with higher mortality in patients with MDA5-ILD (24). Our study showed that the level of MMP7 increased in patients with DM/PM-ILD, but an association with mortality had not been found probably due to racial differences and the small sample size. We firstly showed that age and higher SPD level were significant risk factors for predicting 3-month mortality in MDA5-ILD. The results offered a consideration for disease stratification to early intervene in patients with MDA5-ILD who show worse prognosis. Additionally, therapies for MDA5-ILD targeting SPD-associated pathways may be considerable in future and further researches are required.

The study has several limitations. First, it is a study from one centre with small

sample size. Second, three rare anti-ARS antibodies including anti-KS, anti-Zo and anti-YRS were not routinely tested in our centre, which would result in the underestimating of ARS-ILD. Third, diagnosis of ILD in our cohort was based on respiratory symptoms and HRCT presence and few patients went through lung biopsies, which limited our ability to deeply compare their expressions of biomarkers in lung tissues between MDA5-ILD and ARS-ILD. Further prospective studies are essential to validate the correlation of serum biomarkers in the mechanism of DM/PM-ILD.

In conclusion, we showed a combination of serum biomarkers was also associated with DM/PM-ILD, with differential levels of MMP7 and SPD between patients with MDA5-ILD and ARS-ILD. Age and an increased serum SPD level were the significant predictors for 3-month mortality in patients with MDA5-ILD. Our study provided clues to understand the mechanisms of the diseases and suggested new perspectives for further clinical trials on MDA5-ILD.

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