Cysteine-rich protein 61 as a novel biomarker in systemic lupus erythematosus-associated pulmonary arterial hypertension

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Abstract

Objective

This study aimed to evaluate the level of plasma Cysteine rich 61 (Cyr61) in systemic lupus erythematosus (SLE)-associated pulmonary arterial hypertension (PAH) patients, and to explore the diagnostic and prognostic value of Cyr61 in SLE-PAH.

Methods

Plasma samples were obtained from 54 patients with definite SLE-PAH, 52 SLE-non-PAH patients and 54 healthy controls. Enzyme-linked immunosorbent assay was used to measure plasma Cyr61 concentration, and immunohistochemistry assay was adopted to identify Cyr61 protein expression in lung tissues of monocrotaline (MCT) induced PAH rats at different stages.

Results

Plasma Cyr61 concentration in SLE-PAH patients was significantly higher than matched SLE-non-PAH patients and healthy controls. The optimal cut-off value of Cyr61 in predicting the presence of PAH in entire SLE was 140.7 pg/ml. Further multivariate logistic regression analysis revealed that Cyr61 level≥140.7 pg/ml was an independent risk factor for developing PAH in SLE patients. Kaplan-Meier analysis indicated that SLE-PAH patients with Cyr61 level ≥140.7 pg/ml had better survival than those with lower Cyr61 level (p=0.001 by Log-Rank test), and this was also confirmed by multivariate Cox regression analysis. In addition, Cyr61 protein expression was significantly higher in lung tissue of MCT induced PAH rats compared to control rats, and the expression was more significant in early-mid stage of PAH development than the late stage.

Conclusion

Plasma Cyr61 level was significantly higher in SLE-PAH patients. Elevated circulating Cyr61 is a useful biomarker for identifying PAH in SLE, and it may serve as a promising indicator of prognosis in SLE-PAH.

Key words

systemic lupus erythematosus, pulmonary arterial hypertension, cysteine-rich protein 61, biomarker, prognosis

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder with multi-system involvement (1). Pulmonary arterial hypertension (PAH) is a devastating cardiopulmonary complication of SLE that leads to severe morbidity and mortality. And it has been identified as the third leading cause of mortality in patients with SLE (2). The risk of death for patients with SLE-PAH is far higher than that of the whole SLE population (pooled 5-year survival is 68% *vs*. 94.8%, respectively) (3, 4). Different from PAH patients in western countries, SLE instead of systemic sclerosis is the major underlying type of connective tissue disease-associated PAH in Asian countries (5, 6). In addition, many studies have shown that SLE-PAH patients had a relatively good response to glucocorticoids and immunosuppressants (7). However, due to the poorly described pathological mechanism and rarity of SLE-associated PAH, it is extremely lack of effective clinical signs and laboratory tests that could indicate the presence of PAH in SLE patients. Therefore it seems to be vital to find out more biomarkers, which may potentially be helpful in the early diagnosis, follow-up, and management of SLE-PAH.

Cysteine rich 61 (Cyr61, also called CCN1), a secreted matricellular protein with a molecular weight of 42kd, is encoded by a growth factor-inducible immediate-early gene. It is remarkably versatile and regulates not only angiogenesis, chondrogenesis, and wound healing, but also cell proliferation, adhesion, and migration (8, 9). More importantly, Cyr61 is considered as a novel inflammatory modulator, which has been implicated in leukocyte migration and inflammatory process. Thus, Cyr61 has been extensively studied in many diseases such as cancer, diabetes, inflammatory diseases, cardiovascular diseases, ophthalmic diseases, rheumatoid arthritis and so on (9). Nevertheless, the role of Cyr61 in pulmonary diseases, especially PAH has not yet been elucidated clearly. Our previous study has demonstrated Cyr61 was highly expressed in PAH patients as well as monocrotaline (MCT) induced PAH rats, and promoted the proliferation of pulmonary arterial smooth muscle cells (PA-SMC) via AKT signaling pathway (10). Moreover, Lee *et al*. also revealed that Cyr61 was over-expressed in hypoxia-induced PAH mice, and could suppress PA-SMC contraction in response to hypoxia (11). Except for aforementioned basic researches, there is nearly no literature discussing the value of circulation Cyr61 in PAH patients. Therefore, in this study, we tried to explore the value of Cyr61 in identifying PAH in SLE patients, and to determine the relationship of plasma Cyr61 level and the prognosis in SLE-PAH patients.

Methods

Study population

This was a retrospective study with longitudinally collected data. Subjects were enrolled from Jan 2010 to Jun 2015 at the department of Rheumatology in two tertiary medical centres in Beijing, China. 54 patients with definite SLE-PAH, 52 SLE-non-PAH patients with matched age, gender, SLE duration and SLEDAI, and 54 healthy with age- and gender-matched controls were recruited in this study. All 106 SLE patients fulfilled the 1997 revised American College of Rheumatology criteria for the diagnosis of SLE (12). The diagnosis of PAH was confirmed by right heart catheterisation (RHC) with the criteria of a mean pulmonary arterial pressure (mPAP) ≥25 mmHg together with a pulmonary capillary wedge pressure (PAWP) ≤15 mmHg and pulmonary vascular resistance (PVR)>3 Wood Units (13). While patients with SLE-non-PAH were recruited according to ultrasound cardiography (UCG) with estimated pulmonary artery systolic pressure (PASP)<40 mmHg, and meanwhile the age, gender, SLE duration and SLEDAI score were matched with patients with SLE-PAH. In addition, patients with conditions known to influence Cyr61 levels were excluded including other autoimmune diseases, cancer, diabetes mellitus, active infection, liver diseases and coronary heart disease. Patients with conditions which could induce other types of pulmonary hypertension (PH) including interstitial

lung disease and chronic pulmonary thromboembolism were also excluded. Plasma samples were obtained, split into aliquots, and then stored at -80°C until used.

This study was approved by Institutional Medical Ethics Review Boards of both Peking University First Hospital and Peking Union Medical College Hospital, and all clinical investigations were conducted according to the principles of the Declaration of Helsinki. Informed written consent was obtained from all study participants.

Animal experiments were performed according to the protocols approved by the Institutional Authority for Laboratory Animal Care of Peking University (Beijing, China) and the guide for care and use of laboratory animals published by the US National Institutes of Health.

Subject baseline characteristics

The enrol time of SLE-PAH patients was defined as the time of PAH diagnosed by RHC. Completed medical histories, physical examinations, laboratory tests and plasma samples at baseline visit were collected. In addition, we evaluated PAH-associated data, including WHO functional class (WHO Fc), 6-minute walking distance (6MWD), serum brain natriuretic peptide (BNP) level, serum N terminal-pro brain natriuretic peptide (NT-proBNP) level and haemodynamic parameters. For SLE-non-PAH patients, clinical characteristics and plasma samples were collected at the time point when their age, SLE disease duration and SLEDAI were almost matched with those of SLE-PAH patients.

Follow-up and outcomes

All patients received a comprehensive evaluation every 3 to 6 months. Clinical data including SLE-associated parameters (anti-dsDNA, complements, ESR, CRP and SLEDAI) and PAH-associated parameters (WHO Fc, 6MWD, serum BNP/NT-proBNP level, and UCG) were recorded.

The primary end point was all-cause death. Mortality status was ascertained from medical records as well as telephone interview. The secondary end point was to achieve the integrated treatment goal of PAH recommended by the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) (13). This treatment goal includes the following aspects: 1) clinical symptom: no signs of right heart failure, syncope and progression; 2) exercise capacity: WHO Fc I or II, and 6MWD>380-440m; 3) serology: BNP<50ng/L or NT-proBNP<300ng/L; 4) cardiac imaging: normal right atria area by echocardiography or cardiac magnetic resonance imaging. Treatment goal achieving (TGA) was ascertained according to the follow-up data reported in medical records. All-cause mortality and TGA were set as the long-term and medium-term end-points respectively. The follow-up period was from the date of blood sampling to death, TGA or 30 June 2016.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cyr61 concentration in undiluted plasma samples were measured by enzyme-linked immunosorbent assay (ELISA) method according to manufacturer's instructions (DY4055, R&D Systems, MN, USA). Each sample was tested in duplicate. The absorbance was measured at 450 nm, and plasma Cyr61 concentration was calculated according to a standard curve.

Protocols for animal experiment of monocrotaline (MCT) induced PAH in rats and immunohistochemical staining

Briefly, Sprague-Dawley rats (6 weeks of age, weight 180 to 200 g) were administered a single subcutaneous injection of 60 mg/kg MCT or saline. Rats (n=6 for each group) were anaesthetised at 3, 7, 14, 21 and 28 days after injection of MCT or saline, then RHC was performed to measure the pulmonary arterial pressure, and slides of formalin-fixed, paraffin-embedded lung tissue were prepared for immunohistochemical analysis (10). After deparaffinised, the tissues were stained overnight with primary anti-Cyr61 antibody (Abcam, USA) in a 1:300 dilution at 4°C, and then with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. For colour reactions, diaminobenzidine was used and counter stained with haematoxylin. The staining was analysed by using Zeiss Axio Scan Z1 microscope and Image Pro-Plus image software.

Statistical analysis

The normal distribution test was conducted firstly for all quantitative data. Then quantitative data were presented as mean ± standard deviation if the data were normally distributed, or expressed as median and interquartile range if the data did not follow Gaussian distributions. Categorical variables are described as percentages. In addition, quantitative data were analysed using independent *t*-tests (for parametric data) or Mann-Whitney U-tests (for non-parametric data). Categorical data were compared using chi-squared tests and Fisher's exact tests. Spearman's rank correlation coefficient was used to examine the relationship between the plasma concentration of Cyr61 and other continuous variables. Receiver operating characteristic (ROC) curves were used to examine the usefulness of circulating Cyr61 concentrations in discriminating SLE patients with PAH from those without PAH. Logistic regression analysis was used to determine risk factors for identifying the development of PAH in SLE. Cumulative probabilities of survival and treatment goal un-reaching were calculated by Kaplan-Meier analysis with comparisons performed using the log-rank test. Factors associated with clinical outcomes (death and TGA) were analysed by univariate and multivariate Cox regressions. For all statistical analyses, 2-tailed $p<0.05$ was considered statistically significant. Data analyses were conducted by SPSS 22.0 (SPSS Inc, Chicago, IL).

Results

Demographics and baseline characteristics of all participants

In this study, 54 SLE-PAH patients were enrolled. There was a marked female predominance (96.3%). The average age was 32.2±9.8 years old, with a mean SLE duration of 6.4±5.3 years and a median SLEDAI score of 4 (2, 6) at baseline. Because there were two

missing SLEDAI data in SLE-PAH group, we selected 52 SLE-non-PAH patients with matched age, gender, SLE duration and SLEDAI as controls during the same period, and 49 (94.2%) of them were females. The mean age of this group was 32.1±7.7 years old with a mean SLE duration of 5.4±5.3 years and a median SLEDAI score of 3 (2, 6). A comparison of clinical characteristics between SLE patients with and without PAH showed that the incidence of serositis was higher in the SLE-PAH group (50.0% *vs*. 13.5%, $p<0.001$), and the incidences of malar rash (46.1% *vs*. 37.0%, $p=0.069$) and lupus nephritis (40.4% *vs*. 31.5%, $p=0.092$) had a trend of being higher in the SLE-PAH group. In addition, the percentage of positive anti-nRNP antibody, serum uric acid level and red cell distribution width (RDW-CV) were significantly higher in SLE-PAH group (59.3% *vs*. 25.0%, $p<0.001$; 370.2±101.8 *vs*. 308.9±87.3, $p=0.011$; and 14.4 (13.8, 16.0) *vs*. 13.7 (12.9, 14.6), $p=0.026$, respectively). The other parameters were comparable between SLE with and without PAH groups. There were also 54 age- and gender-matched healthy controls in this study. All of them were ensured to be free of any disease and medications with a mean age of 34.9±8.4 years old, and 52 (96.3%) of them were females. The baseline clinical and laboratory characteristics were shown in Table I.

Table I. Demographics and baseline clinical characteristics.

Features	Healthy controls (n=54)	SLE-non-PAH (n=52)	SLE-PAH (n=54)	*p*-value
Age (years)	34.9 ± 8.4	32.2 ± 9.8	32.2 ± 7.7	0.180
Gender (F/M)	52/2	49/3	52/2	0.836
Cyr61 level (median, quartile)	58.2 (28.9, 80.4)	124.9 (104.1,154.7)	172.5 (143.8,218.2)	<0.001
Clinical characteristics				
SLE duration, years	–	5.7 ± 5.3	6.4 ± 5.3	0.428
Haemoglobin, g/L	–	123.5 ± 17.16	130.0 ± 19.36	0.122
RDW-CV (%)	–	13.7 (12.9, 14.6)	14.4 (13.8, 16.0)	0.026
UA, umol/L		308.9 ± 87.3	370.2 ± 101.8	0.011
ALT, U/L	–	21 (11, 39)	24 (15, 47)	0.171
Creatinine, umol/L	–	58 (47, 69)	62 (53, 73)	0.086
Anti-dsDNA positive (n,%)	–	17 (32.69%)	15 (27.78%)	0.387
Anti-nRNP positive (n,%)	–	13 (25.0%)	32 (59.3%)	<0.001
C3, g/L	–	0.76 ± 0.23	0.79 ± 0.30	0.916
C4, g/L	–	0.14 ± 0.09	0.12 ± 0.07	0.184
SLEDAI, (median, quartile)	–	3 (2,6)	4 (2,6)	0.746
SLE manifestations (n, %)				
Renal involvement	–	21 (40.4%)	17 (31.5%)	0.092
Nervous involvement	–	3 (5.8%)	2 (3.7%)	0.482
Haematological involvement	–	28 (53.8%)	35 (64.8%)	0.311
Serositis	–	7 (13.5%)	27 (50.0%)	<0.001
Malar rash	–	23 (46.1%)	20 (37.0%)	0.069
Arthritis	–	26 (50.0%)	33 (61.1%)	0.170
UCG				
PASP, mmHg	–	27.40 ± 6.93	73.15 ± 19.80	<0.001
RV diameter, mm	–	17.60 ± 3.25	31.18 ± 7.95	<0.001
Pericardial effusion (n, %)	–	5 (9.6%)	18 (33.3%)	0.004
Medications	–			
Glucocorticoids (n, %)	–	52 (100%)	54% (100%)	1.000
Immunosuppressants (n, %)	–	43 (82.7%)	41 (77.4%)	0.390

SLE: systemic lupus erythematosus; PAH: pulmonary arterial hypertension; RDW-CV: red cell distribution width (coefficient of variation); UA: uric acid; ALT: alanine transaminase; anti-dsDNA: anti-double strand DNA antibody; anti-RNP: anti-ribonucleoprotein antibody; C3/4: complement 3/4; SLEDAI: SLE Disease Activity Index; UCG: ultrasound cardiography; RASP: right atrium systolic pressure; RV: right ventricle; *p*-value means SLE-non-PAH *vs*. SLE-PAH.

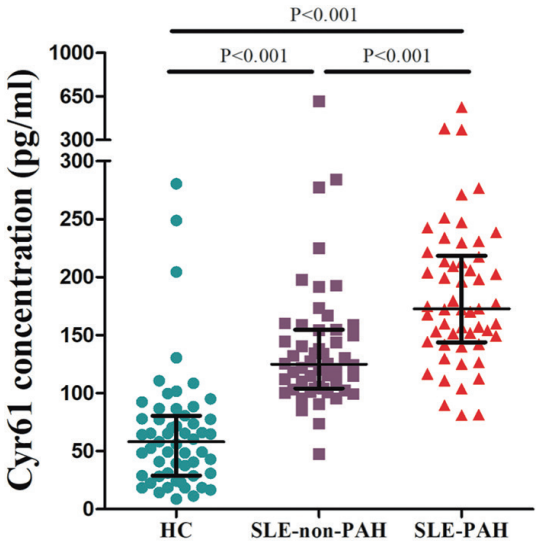


Fig. 1. Concentration of plasma Cyr61 in three groups. Cyr61 concentration in plasma from healthy controls (HC, n=54), SLE-non-PAH (n=52) and SLE-PAH patients (n=54) were determined by ELISA.
SLE-PAH had significantly higher level of plasma Cyr61 than healthy controls (172.5 [143.8, 218.2] *vs*. 58.17 [28.9, 80.4] pg/ml, $p<0.001$) and matched SLE-non-PAH patients (172.5 [143.8, 218.2] *vs*. 124.9 [104.1, 154.7], $p<0.001$).
Additionally, plasma Cyr61 level in SLE-non-PAH patients was higher than HC ($p<0.001$). Horizontal and error bars indicate median value and interquartile range.

Plasma Cyr61 concentrations and its relationship with SLE disease activity

SLE-PAH patients had a significantly higher plasma Cyr61 concentration than SLE-non-PAH patients and healthy controls (median [IQR]: 172.5 [143.8, 218.2]), 124.9 [104.1, 154.7], 58.2 [28.9, 80.4] pg/ml, respectively, $p<0.001$) (Table I and Fig. 1). We noticed that the level of plasma Cyr61 was also elevated in SLE-non-PAH group compared with healthy controls. However, further spearman's correlation analysis were conducted and no significant correlation was found between Cyr61 level and indices of SLE disease activity (including: SLEDAI, C3 and C4 levels) in either SLE-non-PAH group or SLE-PAH group (Fig. 2). Thus, the significantly higher level of Cyr61 in SLE-PAH group was more likely to be associated with the existence of PAH rather than SLE disease severity.

Receiver-operating characteristic (ROC) curve analysis of Cyr61 level in SLE patients

The ROC curve analysis showed the ability of plasma Cyr61 concentration

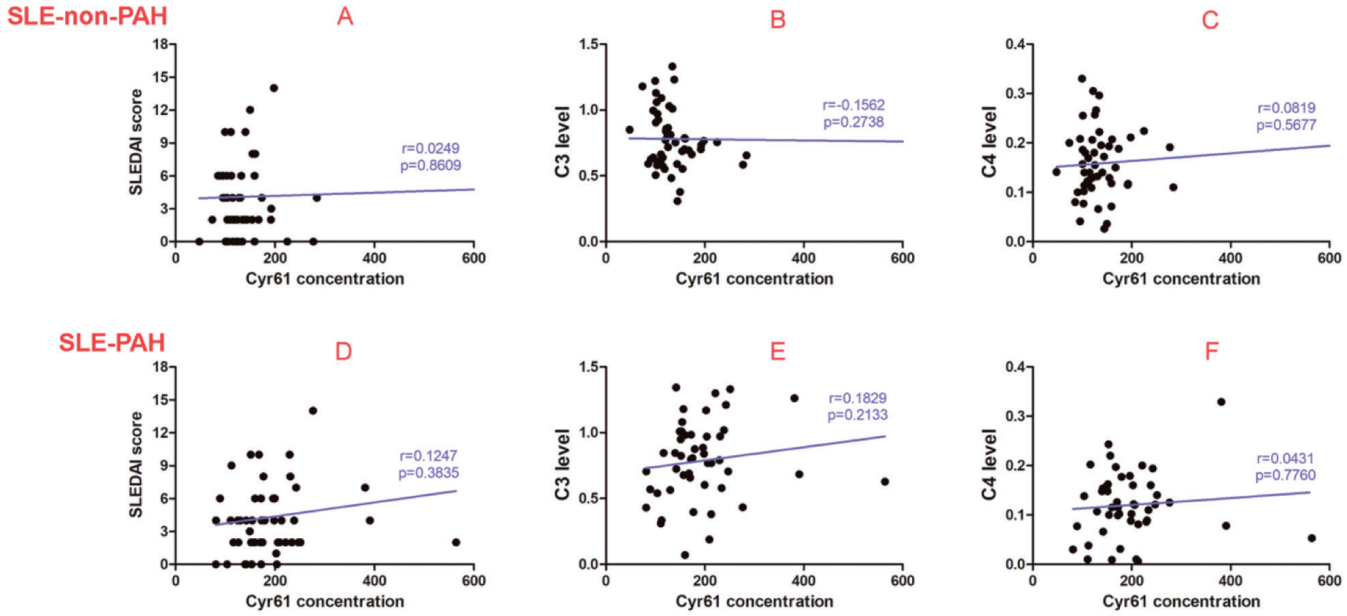
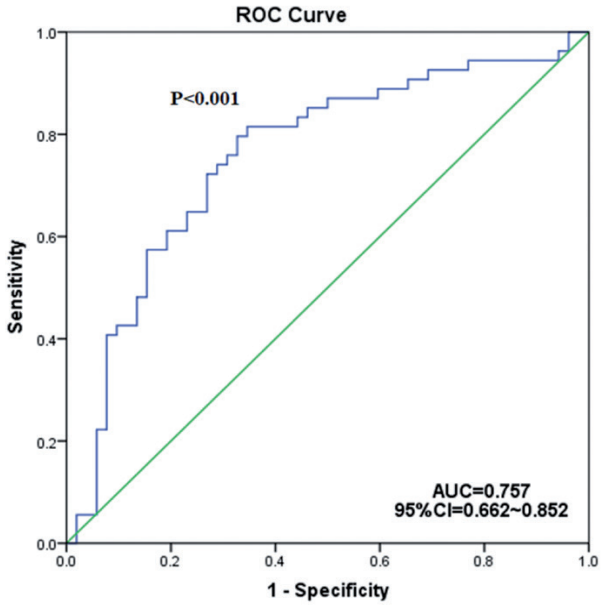


Fig. 2. Correlations between Cyr61 concentration and indices of SLE disease activity in SLE-non-PAH group and SLE-PAH group.
A: SLE Disease Activity Index (SLEDAI) in SLE-non-PAH group; **B**: complement 3 (C3) in SLE-non-PAH group; **C:** complement 4 (C4) in SLE-non-PAH group; **D:** SLEDAI in SLE-PAH group; **E:** C3 in SLE-PAH group; **F:** C4 in SLE-PAH group. There were no significant correlations between Cyr61 concentration and SLEDAI, C3 and C4 in either SLE-non-PAH group or SLE-PAH group.

in discriminating SLE-PAH from SLE-non-PAH with an area under curve of 0.757 (95% CI: 0.662-0.852, $p<0.001$), which represented Cyr61 was a valuable indication of PAH in SLE population (Fig. 3). The optimal cut-off value of Cyr61 was 140.7 pg/ml, with the sensitivity of 79.6%, the specificity of 67.3%, the positive predictive value (PPV) of 70.5% and the negative predictive value (NPV) of 75.6%.

Fig. 3. Receiver operating characteristic (ROC) analysis.
ROC analysis shows the ability of Cyr61 concentration in discriminating SLE-PAH (n=54) from SLE-non-PAH (n=52) with an area under curve of 0.757 (95% CI: 0.662-0.852), $p<0.001$.



Associated variables for identifying PAH in SLE patients

To determine more associated variables for discriminating PAH in SLE patients, univariate and multivariate logistic regression analysis were conducted based on the results of Table I and clinical implication. Variables, including Cyr61≥140.7 pg/ml (cut-off value), UA≥360 umol/L, RDW-CV (per 1 unit), positive anti-nRNP antibody, serositis and presence of UCG pericardial effusion were found to be risk factors in univariate logistic regression analysis. Further multivariable logistic analysis showed that Cyr61≥140.7 pg/ml (OR 20.836, 95% CI: 3.266–132.929, $p=0.001$), RDW-CV (OR 1.344, 95% CI: 1.029–1.754, $p=0.030$), positive anti-nRNP (OR 8.307, 95% CI: 1.771–38.970, $p=0.007$) and presence of UCG pericardial effusion (OR 33.569, 95% CI: 4.930–141.662, $p=0.002$) were independent factors for identifying PAH in SLE patients (Table II).

Comparisons of clinical characteristics between sub-groups of SLE-PAH patients according to plasma Cyr61 concentration

In all SLE-PAH patients (n=54), RHC revealed that the mPAP was 45.26±9.15 mm Hg, the mean CI was 2.88±0.81 L/min/m^2, and the mean PVR was 9.99±4.11 Wood units. Patients with WHO Fc I/II accounted for 53.7%, and the average 6MWD was 446.92±87.63 m. The average BNP and NT-proBNP levels were 135.5 (37.0, 286.0) ng/L and 1174.72±1231.34 pg/ml, respectively (Table III).

To further explore the value of Cyr61 in prognosis of SLE-PAH patients, we then divided SLE-PAH into two subgroups: Cyr61 positive and negative groups by using 140.7 pg/ml as the cut-off value. Comparison was performed

Table II. Univariate and multivariate Logistic regression analysis for identifying PAH in entire SLE patients (n=106).

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
Cyr61≥140.7pg/ml	8.048 (3.338-19.402)	**<0.001**	20.836 (3.266-132.929)	**0.001**
SLE duration	1.045 (0.967-1.128)	0.266	–	
UA≥360 umol/L	3.800 (1.402-10.301)	**0.009**	–	
RDW-CV	1.311 (1.008-1.706)	**0.044**	1.344 (1.029-1.754)	**0.030**
Anti-dsDNA positive	1.126 (0.576-1.682)	0.641	–	
Anti-nRNP positive	7.400 (3.106-17.633)	**<0.001**	8.307 (1.771-38.970)	**0.007**
C3 level	1.086 (0.239-4.930)	0.915	–	
C4 level	1.034 (0.472-1.671)	0.431	–	
SLEDAI	1.010 (0.926-1.102)	0.822	–	
Renal involvement	1.074 (0.871-1.164)	0.618	–	
Malar rash	0.802 (0.368-1.749)	0.579	–	
Serositis	6.429 (2.465-16.764)	**<0.001**		
Pericardial effusion*	9.708 (2.687-35.190)	**0.001**	33.569 (4.930-141.662)	**0.002**

*means presence of pericardial effusion detected by ultrasound cardiography.

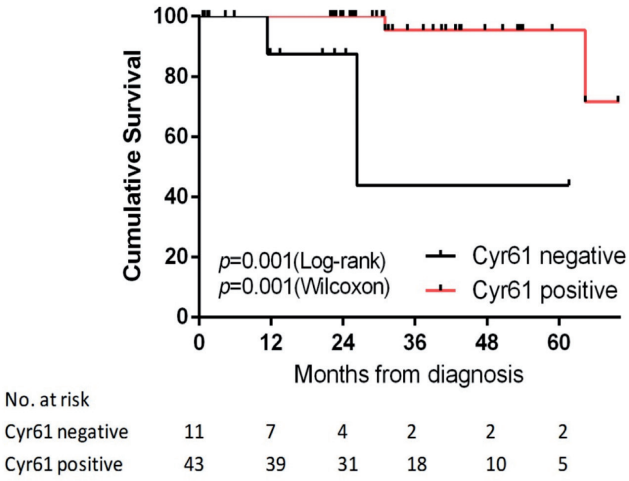


Fig. 4. Cumulative survival in SLE-PAH patients. Kaplan-Meier survival analysis shows the survival in the first decade following PAH diagnosis in SLE-PAH patients with Cyr61 positive and negative groups. Cyr61 positive group (plasma concentration of Cyr61 ≥140.7 pg/ml, red line) had significantly higher survival than Cyr61 negative group (plasma concentration of Cyr61 <140.7 pg/ml, black line) (p=0.001 by Log-Rank test).

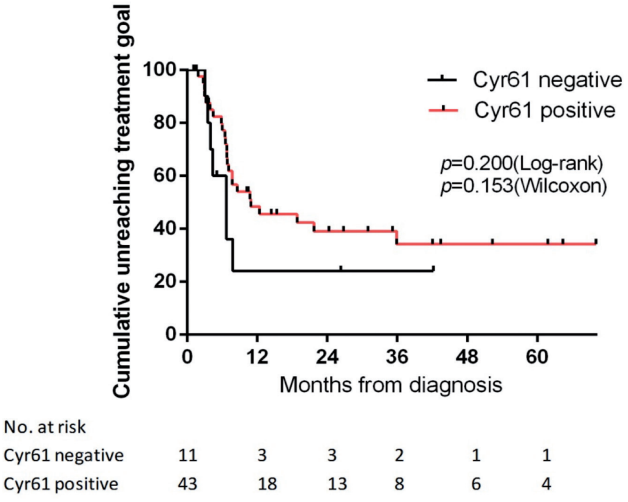


Fig. 5. Cumulative rate of treatment goal un-reaching in SLE-PAH patients. Kaplan-Meier survival analysis shows the rate of treatment goal un-reaching in the first decade following PAH diagnosis in SLE-PAH patients with Cyr61 positive (plasma concentration of Cyr61≥140.7 pg/ml, red line) and negative groups (plasma concentration of Cyr61 <140.7 pg/ml, black line). There was no significant difference between the two groups (p=0.200 by Log-Rank test).

and no significant differences were found between the two sub-groups in terms of clinical characteristic, RHC and UCG parameters and treatment strategies (Table III). However, there was a trend that Cyr61 negative group had pericardial effusion more frequently, less frequent immunosuppressants use and higher death rate than Cyr61 positive group (Table III).

Risk factors related to mortality and TGA in SLE-PAH patients

During an average of 1.77 years follow-up, only 4 (7.4%) deaths occurred (2 in Cyr61 negative group and 2 in Cyr61 positive group). There was no PAH related transplantation or atrial septostomy. Kaplan-Meier analysis of cumulative survival indicated that SLE-PAH patients with negative Cyr61 concentration (<140.7 pg/ml) at baseline had a significantly poorer survival than those with positive Cyr61 level over time (Fig. 4). The death rates were 18.2% and 3.7% respectively in the two sub-groups.

Following univariate and multivariate Cox analyses confirmed that positive Cyr61 was an independent protective factor for mortality with the HR of 0.034 (p=0.018) (Table IV).

TGA was set as the medium clinical end-point. 59.3% of SLE-PAH patients achieved TGA during follow-up. Kaplan-Meier analysis displayed insignificant difference between Cyr61 positive and negative groups in terms of TGA (Fig. 5). Univariate Cox analysis showed that longer PAH duration, higher mPAP, PVR>10 wood units and bigger RV diameters at baseline were adverse factors for achieving TGA status. Unfortunately, no factor was identified to be an independent predictor for TGA by multivariate analysis (Table V).

Dynamic Cyr61 expression in MCT-PAH rat lung tissue

We conducted an animal experiment to explore the dynamic expression of Cyr61 in lung tissue of MCT induced PAH (MCT-PAH) rats and to seek the relationship of Cyr61 expression with PAH severity. Considering of the paracrine/autocrine and external secretion manner of Cyr61, we analysed Cyr61 expression in the whole lung. Immunohistochemical staining of the lung tissue from MCT-PAH rats showed Cyr61 was widely expressed in lung tissue, especially in bronchial epithelium and pulmonary artery walls. Further dynamic studies revealed that Cyr61 expression in rat lung tissue was significantly increased compared with normal control rats from day 3 after MCT injection, continuing elevation to day 7 and peaked on day 14. The expression

Table III. Comparisons of clinical characteristics in SLE-PAH patients according to Cyr61 concentration (n=54).

Variables	Overall (n=54)	Plasma Cyr61 level (pg/ml) Negative group <140.7 (n=11)	Positive group ≥140.7 (n=43)	*p*-value
Age (years)	32.2 ± 7.7	30.3 ± 6.1	32.7 ± 8.2	0.349
SLE duration (years)	6.4 ± 5.3	5.4 ± 5.7	6.6 ± 5.3	0.513
PAH duration (years)	1.8 ± 1.9	1.1 ± 0.8	1.9 ± 2.1	0.197
SLEDAI	4 (2,6)	4 (0,4)	4 (2,6)	0.413
Median Cyr61 concentration	172.5 (143.8,218.2)	112.5 (89.5,126.5)	197.2 (159.2,230.4)	0.001
RHC				
mPAP, mmHg	45.26 ± 9.15	46.91 ± 7.79	45.05 ± 9.51	0.552
PAWP, mmHg	6.58 ± 3.07	7.83 ± 4.32	6.29 ± 2.68	0.154
PVR, WU	9.99 ± 4.11	10.76 ± 4.31	9.81 ± 4.10	0.517
CI, L/min×m^2	2.88 ± 0.81	2.94 ± 0.89	2.85 ± 0.80	0.764
WHO function				
I-II	29 (53.7%)	6 (54.5%)	23 (53.5%)	0.973
III-IV	25 (46.3%)	5 (45.5%)	20 (46.5%)	
Pulmonary function				
FVC (% Pred)	78.44 ± 15.02	84.15 ± 17.13	77.17 ± 14.56	0.311
DLCO (% Pred)	60.84 ± 15.31	67.47 ± 16.38	59.32 ± 14.96	0.246
FVC/DLCO	1.33 ± 0.28	1.29 ± 0.30	1.33 ± 0.27	0.719
UCG				
PASP, mmHg	73.15 ± 19.80	75.64 ± 18.67	72.45 ± 20.28	0.642
RV diameter, mm	31.18 ± 7.95	29.40 ± 3.75	31.69 ± 8.76	0.429
Pericardial effusion	18/44 (40.9%)	6/9 (66.7%)	12/35 (34.3%)	0.128
BNP, ng/L	135.5 (37.0,286.0)	106.0 (34.0,482.8)	142.5 (38.0,284.5)	0.985
NT-proBNP, pg/ml	1174.72 ± 1231.34	943.00 ± 850.49	1249.20 ± 1335.52	0.261
6MWD, m	446.92 ± 87.63	436.90 ± 116.28	449.78 ± 79.50	0.687
Uric acid , umol/L	370.22 ± 101.77	372.00 ± 106.24	369.83 ± 102.52	0.960
RDW-CV (%)	15.03 ± 1.86	14.23 ± 1.16	15.22 ± 1.95	0.175
Medications				
Glucocorticoids (n, %)	54 (100%)	11 (100%)	43 (100%)	1.000
Immunosuppressants (n, %)	41 (75.9%)	6 (54.5%)	35 (81.4%)	0.109
PAH targeted therapy (n, %)				
0	17 (31.5%)	4 36.4%)	13 (30.2%)	0.380
1	33 (61.1%)	7 (63.6%)	26 (60.5%)	
≥2	4 (7.4%)	0 (0%)	4 (9.3%)	
Organ involvement				
Renal involvement	17 (31.5%)	4 (36.4%)	13 (30.2%)	0.726
Nervous involvement	2 (3.7%)	0 (0%)	2 (4.7%)	1.000
Haematological involvement	35 (64.8%)	5 (45.5%)	30 (69.8%)	0.166
Serositis	27 (50.0%)	7 (63.6%)	20 (46.5%)	0.501
Malar rash	20 (37.0%)	3 (27.3%)	17 (39.5%)	0.351
Arthritis	33 (61.1%)	6 (54.5%)	27 (62.8%)	0.733
Death (n, %)	4 (7.4%)	2 (18.2%)	2 (3.70%)	0.181
TGA (n, %)	32 (59.3%)	7 (63.6%)	25 (58.1%)	0.945

mPAP: mean pulmonary arterial pressure; PAWP: pulmonary arterial wedge pressure; PVR: pulmonary vascular resistance; CI: cardiac index; FVC: forced vital capacity; DLCO: diffusing capacity of the lungs for carbon monoxide; UCG: ultrasound cardiography; BNP: brain natriuretic peptides; NT-proBNP: N-terminal pro-brain natriuretic peptide; 6MWD: six-minute walk distance; *p*-value means Cyr61 positive group *vs*. Cyr61 negative group.

of Cyr61 then dropped over time from day 14 to 28. Nevertheless, Cyr61 expression on day 21 and 28 after MCT injection remained higher compared with normal controls (Fig. 6). Higher Cyr61 expression was found on the early-to-mid stage of MCT-PAH than the late stage, which revealed a negative correlation between Cyr61 expression and PAH severity.

Discussion

Cyr61 is a newly recognised secreted matricellular protein, encoded by a growth factor-inducible immediate-early gene. It is transcriptionally activated within minutes of stimulation by inflammatory mediators such as growth factors, cytokines, vitamin D3, whereas the encoding gene is expressed at low levels in quiescent cells (14). This secreted molecule can potentially function as a communicator or messenger among epithelial cells, endothelial cells, fibroblasts, smooth muscle cells, and even inflammatory cells, thus it could regulate a wide range of cellular processes and inflammatory processes including cell migration, adhesion, proliferation, survival and production of cytokines (15). The role of Cyr61 in PAH has not yet been explored clearly. Our previous study demonstrated Cyr61 was highly expressed in not only PAH patients but also MCT-PAH rats, and promoted proliferation of PA-SMC *in vitro*, which could aggravate vascular remodelling in PAH (10). Nevertheless, the number of PAH patients was small in that study, and the variety of connective tissue disease (CTD) types enrolled in that study may lead to a great heterogeneity. Interestingly, almost during the same period, Lee *et al*. revealed that Cyr61 was over-expressed in hypoxia-induced PH mice, and could suppress pulmonary vascular smooth muscle contraction in response to hypoxia, acting as a protective role in PAH (11). Considering the limitations and controversial results in previous studies, we initiated this study to explore the diagnostic and prognostic value of Cyr61 in SLE-PAH, which is the most common type of CTD-PAH in East Asia (5, 6).

In the present study, we have demonstrated for the first time that plasma concentrations of Cyr61 in SLE-PAH patients were significantly elevated compared to the matched SLE-non-PAH patients and normal controls. Plasma Cyr61 concentration was shown to have no correlation with SLE disease activity markers including SLEDAI, C3, and C4 in either SLE-non-PAH group or SLE-PAH group. These findings were partially in contrast with a previous report by Lin *et al*. where serum Cyr61 was associated with SLE disease activity (16). We considered this discrepancy may be contributed by the nature of study design and clinical complexity of SLE. Lin *et al*. recruited 110 SLE patients with a mean SLEDAI score 7.18±3.06. Among these patients, only 27.2% had nephropathy which was much lower than Chinese cross-sectional data revealed by Chinese SLE Treatment and

Table IV. Univariate and multivariate Cox proportional hazards model analysis of mortality in SLE-PAH patients (n=54).

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
Age	0.962 (0.815-1.136)	0.651	–	
SLE duration	1.040 (0.862-1.254)	0.681	–	
PAH duration	1.224 (0.826-1.813)	0.313	–	
SLEDAI	0.993 (0.733-1.346)	0.965	–	
mPAP	1.030 (0.919-1.154)	0.609	–	
PVR >10 WU	4.030 (0.393-41.288)	0.240	–	
RV diameter	0.950 (0.602-1.500)	0.827	–	
WHO fc III-IV	2.085 (0.213-20.458)	0.528	–	
Low complement	1.309 (0.077-22.281)	0.852	–	
NT-proBNP >1500 pg/ml	1.733 (0.142-21.131)	0.666	–	
6MWD< 380m	2.053 (0.185-22.784)	0.558	–	
Uric acid	0.997 (0.984-1.010)	0.646	–	
Kidney involvement	0.898 (0.113-7.114)	0.919	–	
Haematological involvement	3.393 (0.472-24.408)	0.225	–	
Serositis	3.326 (0.343-32.215)	0.300	–	
Cyr61≥ 140.7 pg/ml	**0.042 (0.003-0.510)**	**0.013**	**0.034 (0.002-0.564)**	**0.018**

Table V. Univariate and multivariate Cox proportional hazards model analysis of treatment goal achieving (TGA) in SLE-PAH patients (n=54).

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
Age	0.973 (0.923-1.027)	0.323	–	
SLE duration	0.995 (0.924-1.071)	0.897	–	
PAH duration	0.738 (0.554-0.983)	**0.038**	–	
SLEDAI	1.053 (0.983-1.128)	0.141	–	
mPAP	0.934 (0.897-0.971)	**0.001**	–	
PVR >10 WU	0.397 (0.170-0.928)	**0.033**	–	
RV diameter	0.896 (0.819-0.980)	**0.017**	–	
WHO fc III-IV	1.245 (0.614-2.523)	0.543	–	
Low complement	2.089 (0.977-4.463)	0.057	–	
NT-proBNP >1500 pg/ml	0.711 (0.281-1.801)	0.472	–	
6MWD<380m	0.422 (0.126-1.415)	0.162	–	
Kidney involvement	1.258 (0.576-2.748)	0.564	–	
Haematological involvement	1.744 (0.839-3.626)	0.136	–	
Serositis	0.993 (0.491-2.011)	0.985	–	
Cyr61≥ 140.7 pg/ml	0.577 (0.247-1.352)	0.206	–	

Research group (CSTAR). However, in our study, due to the nature of matched case-control study, we had to enrol our SLE-non-PAH patients by matching age, gender, disease duration and SLEDAI score with those SLE-PAH patients. The median SLEDAI score of our SLE patients was about 4 which was much lower than Lin's study. And this was consistent with some previous literatures revealing the development of PAH and its severity did not correlate with SLE disease activity (17, 18). Therefore, it seems to be reasonable that no correlation was found between Cyr61 level and SLE disease severity, indicating it is more important to explore the relationship between Cyr61 and PAH in SLE.

Further ROC curve analysis revealed that Cyr61 could act as a potential biomarker in identifying PAH in SLE patients with an optimal cut-off value of 140.7 pg/ml. In the following logistic regression analysis, positive Cyr61 (≥140.7 pg/ml), positive anti-nRNP antibody, RDW-CV and presence of pericardial effusion in UCG were identified as independent predictors for the presence of PAH in SLE. Among these parameters, presence of anti-nRNP antibody and pericardial effusion have already been widely reported as strong predictors for the development of PAH in SLE (19-22). Several studies have also demonstrated RDW-CV as a predictor for diagnosis and prognosis of other PH types. RDW-CV was bigger in idiopathic PAH and chronic thromboembolic PH patients compared to normal controls and pulmonary thromboembolism without PH patients respectively (23-25). In addition to these recognised predictors, we are now reporting that elevated Cyr61 is an independent biomarker for indicating the presence of PAH in SLE patients.

The relationship between Cyr61 level and prognosis in SLE-PAH patients has never been investigated before. In this study, interestingly, we found that high Cyr61 concentration (≥140.7 pg/ml) at baseline was independently associated with a favourable prognosis by both Kaplan-Meier analysis and Cox regression analyses. In addition, the animal experiments revealed that Cyr61 expression in the lung tissue of MCT-PAH rats was negatively correlated with PAH disease severity. Higher Cyr61 expression by immunohistochemistry assay was seen in the early-mid stage after MCT injection (day 7/14). Coincidently, in our previous research, we found the expression of Cyr61 in the lung tissue of MCT-PAH rats detected by Western Blot assay was also decreased over time from early to the advanced stage of PAH development (10). Admittedly no animal model mimics exactly all the features of human PAH disease. Nevertheless, amongst the several existing PAH experimental models, the monocrotaline (MCT) model is perhaps the one that has most contributed to the understanding of PAH pathophysiology (26, 27).

Although elevated circulation Cyr61 concentration indicates the presence of PAH in SLE patients, the SLE-PAH patients with higher Cyr61 level have better survival than the SLE-PAH patients who do not have. The possible mechanisms behind this negative correlation would be very intricate and yet indistinct. Recently, Lee *et al*. revealed that Cyr61 expression was up-regulated in hypoxia induced PH mice, and furthermore Cyr61 could prevent hypoxia-induced PA-SMC contraction *in vitro*, as well as decrease RV pressure and disease

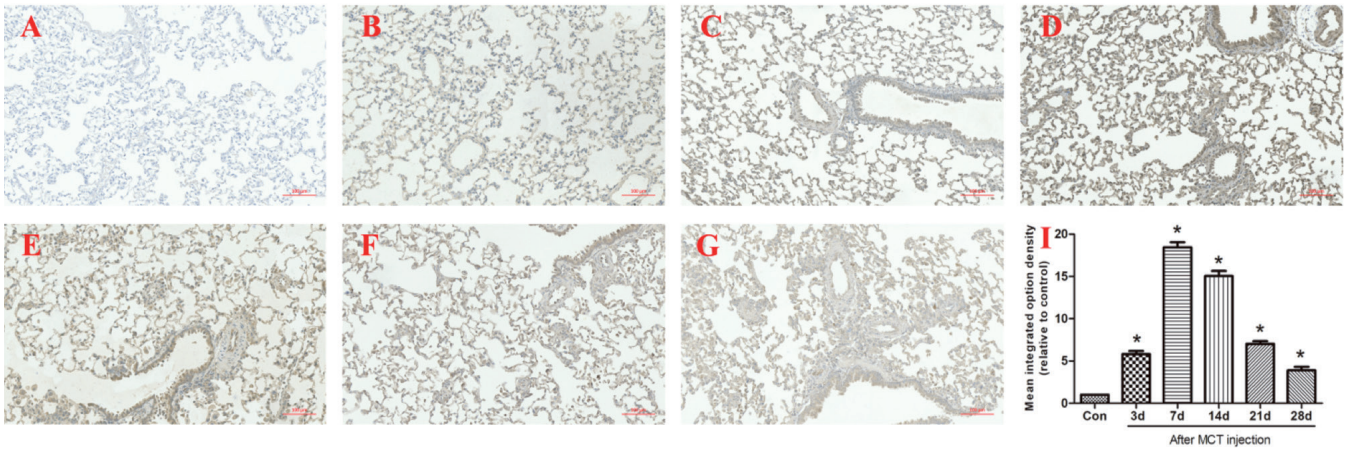


Fig. 6. Expression of Cyr61 in rat lung tissues.
Immunohistochemistry was used to determine the expression of Cyr61 protein in rat lung tissues. Monocrotaline (MCT) induced PAH rat model was set up by a single injection of MCT (60mg/kg) subcutaneously.
A: negative control; **B:** normal control; **C:** 3 days after monocrotaline injection; **D:** 7 days after monocrotaline injection; **E:** 14 days after monocrotaline injection; **F:** 21 days after monocrotaline injection; **G:** 28 days after monocrotaline injection; **I:** mean integrated option density analysed by image pro plus 6.0 software.
Representative immunohistochemical images were shown here. Each figure represents 2 independent experiments, n=6 for each group. Scale bars: 100 μm. *p<0.05 *vs*. control.

progression in PH animals. Besides, in our opinion, there may be two possible explanations for this phenomenon. On the one hand, Cyr61, as a secreted protein encoded by growth factor-inducible immediate-early gene, could be induced right away after inflammatory stimulation (9). Inflammation and dysregulated immunity are now increasingly recognised to be vital in the pathogenesis of PAH. Acute inflammation is more significant in the early stage of PAH development than the advanced stage where tissue repairing and vascular remodelling predominate (28-31). These could explain why Cyr61, as a pro-inflammatory cytokine, was expressed more in the early stage. On the other hand, Cyr61 could induce diverse and sometimes opposing cellular responses through distinct receptors in a cell-type and context-dependent manner. Generally, Cyr61 is known as a double-edged sword in cancer because it could promote cell proliferation, survival and angiogenesis as well as induce apoptosis and cellular senescence. For example, Ladwa *et al*. found higher level of Cyr61 mRNA expression in colorectal cancer compared with normal colons, but the expression was relatively lower in more advanced cancers (32). Mori *et al*. noted that Cyr61 low expression group was clinically more advanced than the Cyr61 high expression group in terms of disease progression in lung cancer (33), which was very similar to what we found in SLE-PAH.

Interestingly, we did not find significant correlation between Cyr61 and haemodynamic variables in SLE-PAH patients. There was no significant difference in haemodynamic variables between positive and negative Cyr61 groups. Actually, the lack of correlation of circulating biomarkers that predict survival outcome in PAH, with haemodynamic variables, has never been odds. For example, Rhodes *et al*. found that reduced microRNA-150 was an independent predictor for poor survival but was not significantly correlated with pulmonary arterial pressure, pulmonary vascular resistance, cardiac output or right atria pressure (34). It is said that such changes in peripheral blood indices may be due to mechanisms that are independent of right ventricular compromise. In addition, article published in *Arthritis & Rheumatology* 2016 also revealed that although haemodynamic parameters were similar in anti-U1 RNP positive and anti-U1 RNP negative patients, anti-U1 RNP positivity was negatively and independently associated with mortality in CTD-associated PAH (35).

It is also important to acknowledge some limitations of our study. Firstly, limited number of patients enrolled in the study due to the rarity of SLE-PAH. However, essentially this is a hypothesis-generating study and we have made cases-controls matched strictly to eliminate bias as much as possible. Secondly, we were also limited by using both incident and prevalent cases and hence different SLE treatment approaches implemented in our patients may influence prognosis. Last but not least, we did unfortunately not find any other risk factors, like 6MWD and NT-proBNP, to predict mortality of SLE-PAH except for Cyr61. We suspected this might be explained by small sample size and relatively favourable prognosis of SLE-PAH in our study. Actually, patients with WHO Fc I/II accounted for 53.7% which was higher than other cohorts (20, 36), and only 4 (7.4%) deaths occurred during an average of 1.77 years follow-up. Thus, it is necessary to validate current supposition by enlarging case number, extending follow-up time and using prospective cohort. In addition, basic studies about molecular mechanisms underlying these conditions are under way.

Conclusion

In summary, in the present study we found for the first time that plasma Cyr61 concentration was elevated in SLE-PAH patients compared to SLE-non-PAH patients and healthy controls. The elevated circulating Cyr61

is a predictor for identifying PAH in SLE patients, and surprisingly, is an independent factor for implicating better prognostic outcome in SLE-PAH patients. The expression of Cyr61 in the lung tissue of MCT-PAH rats was higher in early stage of PAH development, suggesting its potential role of detecting PAH in early stage in SLE patients. In future, additional larger prospective clinical studies as well as basic researches are needed to confirm these findings and to determine whether Cyr61 would be a potential biomarker for the therapy target for PAH.

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