Hook effect and serum KL-6 in Patients with Systemic Sclerosis-Associated Interstitial Lung Disease (SSc-ILD)

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Abstract

Objective To determine whether serum KL-6 can be used as an alternative diagnostic tool for Systemic Sclerosis-associated ILD (SSc-ILD).

Methods: This was a cross-sectional study with compatibility analysis of SSc patients visiting the rheumatology clinic of Dr. Hasan Sadikin General Hospital, Bandung, Indonesia in 2019-2020. Inclusion criteria were aged 18-60 years, diagnosed with SSc, restrictive spirometry results followed by HRCT thorax, having stored biological materials (<2 years) for serum KL-6 examination, and body mass index of <23 kg/m^2. Patients with pneumonia, tuberculosis, history of tuberculosis, malignancy, and cardiac decompensation were excluded. Serum KL-6 level of >397.5 unit/mL was declared as ILD. The compatibility analysis was performed using the Cohen's Kappa test.

Results: Thirty-eight subjects, mostly women (94.7%), with mean age of 39 years participated in this study. Most of the subjects suffered from diffuse systemic sclerosis subtypes (57.9%). Subjects had received cyclophosphamide (10.5%), MMF (2.6%), and other medications. Almost all subjects (97.4%) demonstrated ILD features on HRCT thorax. The median serum KL-6 level was 53.22 Units/mL, which was much lower than the findings in other studies, but the hook effect could not be proven. The Kappa coefficient was found to be 0.003 with a p-value of 0.811.

Conclusion: There is no compatibility between serum KL-6 level and ILD features based on HRCT thorax in SSc-ILD. The presence of hook effect needs more attention.

Keywords: Hook effect, ILD, KL-6, systemic sclerosis

Introduction

Interstitial lung disease (ILD) is a group of diseases that involve the walls and tissues surrounding the alveoli.[1] Damage to the lung parenchyma triggers a fibrotic process that causes severe gas exchange and ventilation disorders. The etiology of this disease varies, among which occurs in 50% of patients with systemic sclerosis (SSc) which is influenced by genetic factors, ethnicity, and environmental exposure.[2] This disease is difficult to diagnose, especially in developing countries due to limited diagnostic facilities.[3] Pulmonary function tests can show restrictive lung abnormalities but cannot differentiate the cause.[2] Radiological examinations such as chest X-ray are inexpensive and easy to perform and have little radiation exposure but can give a normal picture on ILD. Therefore, HRCT thorax is an option for establishing the diagnosis of ILD even though it causes radiation exposure, requires high costs and is difficult to perform due to limited facilities so that routine examinations cannot be carried out to monitor the progress of the disease course in short time intervals, whereas in SSc

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patients with ILD need periodic examinations to assess the progress of ILD and response to therapy.[2, 3]

Another safer and cheaper assay is serum KL-6 which has been known to enhance the chemotactic and anti-apoptotic effects of fibroblast cells.[4] Krebs von den Lungen-6 is an ILD biomarker that has high sensitivity and specificity for diagnosing, monitoring, and predicting prognosis.[5] Thus, serum KL-6 is expected to be an alternative examination for ILD in patients with systemic sclerosis. Despite the specificity, numerous interferences are possible. Interferences can be defined as the effect of substances present in an analytical system which causes deviation of the measured value from the true value. The interferences can cause false increase or false decrease of measured result, which might cause misleading laboratory report.[6] This study aims to determine if serum KL-6 can be used as an alternative diagnostic tool for SSc-associated ILD (SSc-ILD).

Methods

Subjects of this study was SSc outpatient in rheumatology clinic of Hasan Sadikin General Hospital Bandung, Indonesia in 2019-2020. Inclusion criteria were aged 18-60 years, diagnosed with SSc in line with the American college of rheumatology/European league against rheumatism (ACR/EULAR) 2013 classification criteria for SSc, restrictive spirometry results and continued with HRCT thorax, having stored biological material (less than 2 years) for serum KL-6 examination, and body mass index less than 23 kg/m^2. Exclusion criteria were pneumonia, tuberculosis, history of tuberculosis, malignancy or history of malignancy, and functional class III-IV cardiac decompensation.

All patients underwent HRCT thorax examination as gold standard for ILD. The results of the HRCT thorax were grouped based on the presence or absence of ILD features contained in the expertise of the thoracic team of the Radiology Department, Faculty of Medicine, Universitas Padjadjaran/Hasan Sadikin General Hospital Bandung, which were approved by 2 consultants, including features of fibrosis, ground glass opacities, irregular pleural margins, septal or subpleural lines, honeycombing, and subpleural cyst.

Serum KL-6 levels were collected as primary data. Blood samples from all the patients were collected in the same day or 7 days maximum length from HRCT thorax examination. Serum were stored in the refrigerator at -80^{o}C. Serum KL-6 concentrations were measured using an enzyme-linked immunosorbent assay kit (Bioassay Technology Laboratory, China), according to the manufacturer's instructions. Serum KL-6 levels >397.5 unit/mL were declared as ILD, using cut-off from a study in China that met the clinical trial requirements, since there has been no diagnostic study for serum KL-6 in Indonesia.

Data collection began with searching for data on patients with systemic sclerosis at the Hasan Sadikin General Hospital rheumatology clinic for 2019-2020 in the SSc registry. Blood samples of patients who met the inclusion and exclusion criteria were collected in the form of stored biological material at the Department of Clinical Pathology under the SSc Registry research. The length of blood sampling and thorax HRCT examination is a maximum of 7 days. Examination of serum KL-6 levels done with ELISA technique.

This is a cross-sectional study method with diagnostic test evaluation. Sample measurement was determined using the compatibility test formula, with a minimum total sample of 37 subjects was required. Secondary data for baseline characteristic was obtained from Hasan Sadikin General Hospital systemic sclerosis registry, including age, sex, disease duration, medications, and modified Rodnan Skin Score (mRSS).

Number and percentage were used for categorical variables. The continuous variables were tested by Kolmogorov-Smirnov normal distribution. Mean ± standard deviation was used for measurement data following the normal distribution. Median was used for non-normal distribution measurement data. A value of $p<0.05$ suggested the result was statistically significant. Cohen's Kappa test was used to analyze compatibility of serum KL-6 levels with HRCT thorax.

This study was approved by the Health Research Ethics Committee of Dr. Hasan Sadikin General Hospital Bandung with ethical approval number LB.02.01/X.6.5/318/2020. For all patients who were enrolled in this study, the informed consent form was signed by the patient herself/himself.

Result

There were 85 patients with systemic sclerosis recorded in the SSc registry, with 7 people over 60 years old, 33 people having no data of HRCT thorax, 23 people not having BBT. The subjects analyzed in this study were 38

Table 1 Subject Characteristics

Variable	n = 38
Age (years), mean ± SD	39 ± 11
Female, n (%)	36 (94,7)
Disease duration (month), median (range)	24 (4–168)
Overlap syndrome, n (%)	7 (18,4)
Subtype, n (%)	
dSSc	22 (57,9)
lSSc	16 (42,1)
Medications, n (%)	
History of cyclophosphamide	4 (10,5)
History of *Physalis angulata Linn.*	6 (15,8)
Methylprednisolone	33 (86,8)
Methotrexate	27 (71,1)
MMF	1 (2,6)
Azathioprine	3 (7,9)
Cyclosporin	1 (2,6)
MRSS, mean ± SD	17,53±6,425
Serum KL-6 level (Unit/mL), median (range)	53,22 (21,62–444,07)
ILD features in HRCT thorax, n (%)	37 (97,4)

dSSc, diffuse systemic sclerosis; HRCT, High Resolution Computed Tomography; KL-6, Krebs von den Lungen-6; lSSc, limited systemic sclerosis; MMF, *mycophenolate mofetil*; MRSS, *modified Rodnan Skin Score*; ILD, interstitial lung disease. Median serum KL-6 levels in this study was 53.22 Units/mL (range 21.62-444.07 Units/mL), lower than studies in various countries, although the results of HRCT thorax with ILD features were found in almost all study subjects. There were only 3 subjects in this study who had serum KL-6 levels more than 200 Unit/mL.

patients who met the inclusion and exclusion criteria.

Almost all of this study subjects were women. Other baseline characteristics showed some differences from other studies, such as the age of the subjects in this study which was lower than studies in other countries, and the diffuse SSc (dSSc) subtype which was more than the limited SSc (lSSc) subtype. Complete baseline characteristics can be seen in Table 1.

Subgroup analysis was carried out because the results of this study were different from other studies. Subtype and administration of therapy are known to affect serum KL-6 levels. The dSSc subtype was said to have higher serum KL-6 levels than the lSSc subtype. Administration of cyclophosphamide therapy, which is the treatment of choice for pulmonary

Table 2 Compatibility between Serum KL-6 levels (cut off 397,5 Unit/mL) and ILD Features based on HRCT thorax

Variable	Total	ILD		Kappa coefficient	P value
		Yes	No		
		n=37	n=1		
Serum KL-6 levels > 397,5 U/mL					
Yes	2	2	0	0,003 (-0,004 – 0,010)	0,811
No	36	35	1		

HRCT, High Resolution Computed Tomography; KL-6, Krebs von den Lungen-6

Table 3 Compatibility between Serum KL-6 levels (threshold 53,22 Unit/mL) and ILD Features based on HRCT *Thorax*

Variable	Total	ILD		Kappa coefficient	P value
		Yes	No		
		n=37	n=1		
KL-6 > 53,22 U/mL					
Yes	19	19	0	0,053 (-0,049 – 0,154)	0,311
No	19	18	1		

HRCT, High Resolution Computed Tomography; KL-6, Krebs von den Lungen-6

fibrosis, is said to reduce serum KL-6 levels. The result showed that the differences in serum KL-6 levels between the dSSc subtype groups and lSSc, as well as between the group receiving cyclophosphamide therapy and the group without cyclophosphamide therapy were not statistically significant.

Analysis testing using the Cohen Kappa formula was carried out to test the compatibility between serum KL-6 levels and ILD features based on HRCT thorax in SSc patients with restrictive lung disease.

Cohen's Kappa analysis with a cut off value of serum KL-6 levels of 397.5 units/mL showed no agreement, so an analysis was carried out using the median in this study (53.22 units/mL), as can be seen in Table 3.

The Kappa coefficient was in the range of 0.0–0.20 indicating a very low concordance with a p-value>0.05 which indicated that these results were not statistically significant. The results of this study showed that there was no compatibility between serum KL-6 levels and ILD features based on HRCT thorax in SSc-ILD.

Discussion

This is the first study in Indonesia to assess the compatibility between serum KL-6 levels and ILD features based on HRCT thorax in SSc-ILD. The presence of previously unknown interferences needs to be considered because this study showed different results from other similar studies, the results did not match the clinical picture of the study subjects, and did not match the gold standard of imaging results. The interferences can be defined as the effect of the substance contained in the examination that causes the deviation of the measured value from the true value. Interferences in the immunoassay examination can cause false increases or false decreases. The hook effect that can occur in this study corresponds to the saturation curve between analyte (antigen) and antibody. The hook effect indicates the presence of a very large amount of analyte which saturates the binding sites on the antibody. This phenomenon is common in analytes whose serum levels have a very wide range, such as C-reactive protein, hormones, and tumor markers. The hook effect can be proven if the diluted sample shows a higher value than the undiluted sample, which unfortunately could not be done in this study.[6]

Serum KL-6 levels in this study were so low in 36 subjects that they could not be categorized as ILD when using a cut off value of 397.5 Units/mL. These results were not in accordance with the ILD features obtained from almost all HRCT thorax examinations in this study. Another study showed that the examination of serum KL-6 levels had a sensitivity and specificity of above 80% for the incidence of ILDs.[7] The difference of the results in this study, the low serum KL-6 level, indicated a hook effect. The incidence of hook effect on serum KL-6 examination had never been recorded before so it was not listed in the reagent kit. Unfortunately, this study could not prove the hook effect. Examination of serum KL-6 levels without dilution of the sample was a limitation of this study. This phenomenon became a note for further research on serum KL-6 levels, that it is necessary to dilute the sample.

Serum KL-6 levels can be influenced by several factors, including the hook effect as previously discussed, ethnic and genetic differences, ages, SSc subtype, and the administration of therapy. Cut off values for serum KL-6 levels had known different in 2 ethnicities and 3 genotypes, including German and Japanese ethnicities and genotypes A/A, A/G, and G/G.[8] Differences in cut off values for serum KL-6 levels were also found among various Asian populations.[4,7,9,10] This may also explain the difference in mean or median serum KL-6 levels in studies in various

countries, including this study.[4,8]

This study had a mean age of 39 years. The Japanese and Hungarian studies had an older mean age.[11, 12] The differences in mean age show the differences in the characteristics of SSc patients in various countries. Serum KL-6 levels increase with age.[13] Age-related physiological changes occur in the respiratory system, including reduced elastic recoil, chest wall stiffness, changes in gas exchange, and increased flow resistance. These changes contribute to increased morbidity and mortality of lung diseases in the elderly, including chronic obstructive pulmonary disease, lung cancer, pulmonary fibrosis, and infection, which may affect serum KL-6 levels in older individuals.[14]

As many as 52.6% of this study subjects were dSSc, while other studies in China and Hungary were more of the lSSc subtype.[9,11] The difference could be due to differences in the characteristics of SSc patients related to ethnicity and genetics. Interstitial lung disease is found in the lSSc subtype with slow progression and generally mild. Interstitial lung disease in the dSSc subtype was more common, with an earlier onset, faster progression and more severe clinical course, so that the serum KL-6 level in the dSSc subtype was higher than in the lSSc subtype.[15] Serum KL-6 levels in this study should be similar to other studies with more dSSc subtypes, or higher than other studies with more lSSc subtypes.

Administration of therapy to SSc-ILD does not necessarily lead to low serum KL-6 levels. Methotrexate has not shown improvement in pulmonary function on ILD, whereas idiosyncratic drug-related hypersensitivity pneumonitis is known.[16] There was a decrease in serum KL-6 levels after 1 year of administration of cyclophosphamide and MMF with an average decrease of 100.6 units/mL. The use of MMF and cyclophosphamide in this study was only found in a small number of study subjects so that the low serum KL-6 did not thought to be due to the effects of therapy. In addition, serum KL-6 levels after 1 year of cyclophosphamide and MMF therapy remained higher than in healthy subjects.[11, 17]

Several subjects in this study had participated in a randomized, double-blind clinical trial that added *Physalis angulata Linn*. ethanol extract in SSc patients who had received standard therapy conducted by Dewi, *et al*.[18] This study proved that *Physalis angulata Linn.* had a repairing effect on skin fibrosis as assessed by a decrease in MRSS and a decrease in serum fibrosis biomarkers, namely Procollagen Type IN Terminal Propeptide (P1NP). *Physalis angulata Linn.* is known to have therapeutic potential, as anti-inflammatory, antioxidant, immunomodulatory, antiproliferative, and anticancer. Epidemiological data on the use of *Physalis angulata Linn.* and the efficacy of *Physalis angulata Linn.* for pulmonary fibrosis disorders as well as for reducing serum KL-6 levels are still in the research stage.[18] *Physalis angulata Linn.* use was not recorded in this study. However, history of using *Physalis angulata Linn.* can be considered as one of the confounding factors that affect serum KL-6 levels in this study.

Most of the samples taken for examination of serum KL-6 levels and examination of HRCT of the thorax were carried out on the same day, while some examinations were carried out with a maximum length of 1 week. All study subjects were patients who had been diagnosed with SSc who were undergoing outpatient treatment. Sampling of serum KL-6 levels and HRCT thorax examination were carried out on subjects with equally controlled disease activity and not relapse or had infection, according to inclusion and exclusion criteria. Thus, disease activity and infection were not considered as confounding factors that could influence the results of this study.

Another limitation of this study related to the study subjects who were not firstly diagnosed so that they have received therapy, both standard therapy and *Physalis angulata Linn.* In addition, the duration of therapy was not included. In this study, lung fungtion test were carried out using spiromerty. However preferable lung function test for ILD is Diffusing Capacity of the Lungs for Carbon Monoxide (DLCO), not spirometry. DLCO is not used in this study because the device is not available.

Study by Yani, *et al*.[19] at Dr. Hasan Sadikin General Hospital Bandung showed that there was no relationship between serum KL-6 levels with forced vital capacity (FVC) and modified Rodnan Skin Score (mRSS) in patients with restrictive lung disease in diffuse type systemic sclerosis.There was a possibility that serum KL-6 cannot be used to diagnose SSc-ILD in Indonesia population. Several plasma or serum proteins that can be candidate biomarkers to diagnose, monitor, and predict the prognosis of ILD include interleukin-6 (IL-6), C-reactive protein (CRP), CC chemokine ligand (CCL)-2, CCL-18, CXCL4, matrix metalloproteinases

(MMPs) and surfactant protein-D (SP-D).[12, 17, 20]

There is no agreement between serum KL-6 levels and ILD features based on HRCT thorax in SSc-ILD. The hook effect affect serum KL-6 levels so that it cannot be used as an alternative diagnostic test for SSc-ILD. A diagnostic study is suggested to determine cut off value of serum KL-6 levels for patients who are firstly diagnosed SSc-ILD and not received any therapy yet, with dilution during the examination of serum KL-6 levels, in the Indonesian population, in the diffuse SSc subtype.

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