SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2014; 31; 55-61

Decreased sputum caveolin-1 is associated with systemic sclerosis related lung disease

Neslihan Yilmaz[1], Sehnaz Olgun[2], Rengin Ahiskali[3], Sait Karakurt[2], Sule Yavuz[1]

[1]Rheumatology, Marmara University Faculty of Medicine, [2]Chest Medicine, Marmara University Faculty of Medicine, [3]Pathology, Marmara University Faculty of Medicine, Istanbul, Turkey

Abstract. *Aim:* To determine serum and sputum Caveolin-1 (Cav-1) levels and their associations with transforming growth factor- β (TGF-β) and interstitial lung disease (ILD) in systemic sclerosis (SSc). *Methods:* Serum and induced sputum samples from 55 patients with SSc, 25 asthma patients and 16 healthy volunteers (HC) were tested for Cav-1 and TGF-β by the ELISA technique. As a possible downstream signaling regulator of TGF-β, Endothelin-1 (ET-1), a potent profibrotic protein, was also measured in all serum and sputum samples and relations with Cav-1 and TGF-β were sought. All scleroderma patients were evaluated for their clinical and laboratory parameters. Pulmonary function tests (PFT) and high resolution computerized tomography (HRCT) were performed for the diagnosis of ILD. The alveolitis-fibrosis index and the SSc disease severity scores were noted for each patient. *Results:* Serum Cav-1 levels were lower in SSc compared to HC ($p<0.01$). Cav-1 levels were significantly lower in the sputum of SSc patients compared to both control groups ($p<0.001$). It was also found significantly lower in SSc-ILD compared to those without ILD (0.19±0.04 vs 0.25±0.07, respectively, $p<0.01$). Although no difference was found in the serum TGF-β levels among the groups, sputum TGF-β levels correlated positively with the alveolitis index ($r=0.34$) and correlated inversely with FVC measurements ($r=-0.44$, $p<0.05$) among SSc patients. Serum ET-1 was significantly higher in SSc patients ($p<0.01$) but no association was found between ET-1 and Cav-1 or TGF-β. *Conclusion:* These results suggest that decreased sputum Cav-1 levels is associated with SSc related-ILD. Its use as a marker for the detection of SSc-ILD warrants further evaluation. *(Sarcoidosis Vasc Diffuse Lung Dis 2014; 31: 55-61)*

Key words: Scleroderma, lung disease, Caveolin-1

Introduction

Systemic sclerosis is an autoimmune disease, characterized by excessive collagen deposition and microvasculopathy. Pulmonary involvement including ILD has emerged as the leading cause of morbidity and mortality in SSc patients (1). Interstitial lung involvement was reported up to 90% in autopsy series with up to 30% of deaths directly attributable to lung fibrosis (2-4). Although the pathogenesis of scleroderma is still unclear, the overproduction of the extracellular matrix components by fibroblasts is the hallmark feature of affected tissues. Transforming growth factor -β (TGF-β) plays a crucial role in tissue fibrosis and has been implicated in the pathogenesis of SSc. Even though, serum results are contradictory (5-8), increased TGF-β levels has been shown in the bronchoalveolar lavage (BAL) of patients with SSc–ILD (9). Recent evidences suggest that Caveolin-1(Cav-1), the main protein component of caveolae, participates in the pathogenesis of

Received: 07 May 2013
Accepted after revision: 22 August 2013
Correspondence: Sule Yavuz
Bagdat Cad. Trak Apt. 149/3
Selamicesme / Istanbul
Gsm: 00905324647436
E-mail: suleyavuz@gmail.com

fibrotic diseases through regulation of TGF-β receptor (TβR) degradation and activation (10-14). TGF-β leads to a decrease in Cav-1 expression of human lung fibroblasts, whereas, Cav-1 is able to suppress TGF-β induced extracellular matrix production in cultured fibroblasts (12). Moreover Cav-1 deficient mice exhibit several abnormalities including interstitial lung disease, decreased vascular tone, and lipid abnormalities (15). In human studies, Cav-1 expression was shown to be decreased in the lung biopsies of idiopathic pulmonary fibrosis, asthma and SSc patients (10,12,16,17). Endotelin-1 (ET-1) is a peptide that exhibits potent effects on vascular/nonvascular smooth muscle cells and plays a role in SSc pathogenesis by inducing proliferation of endothelial cells and fibroblasts (18). Additionally, recent data suggested that ET-1 not only increase TGF-β and activate TGF-β signaling but it also appears that TGF-β induces ET-1 in human lung fibroblasts(19). It is also known that Endothelin-1 receptors (ET-Rs) interact with Cav-1 and co-localize in caveolae which integrate different receptor and signaling proteins. Indeed, high levels of ET-1 were found in serum samples of SSc patients and also in BAL samples of SSc patients with ILD (20).
Taken together, lung fibrosis is controlled by several factors including Cav-1, however the utility of measurement of Cav-1 in real life has not been fully elucidated. We have recently showed that composition of induced sputum reflects the content of BAL fluid in SSc patients (21). So, we tested the hypothesis that whether or not Cav-1 could be used as a non-invasive marker to determine ILD in patients with scleroderma using serum and sputum samples. To address this issue, Cav-1 levels were measured in serum and induced sputum samples and associations between TGF-β and ET-1 levels and clinical features were assessed.

Materials and methods

Subjects

Fifty-five consecutive patients with SSc who were followed regularly in University of Marmara, Department of Rheumatology, were enrolled in this prospective case-control study. All patients had fulfilled the criteria for as proposed by LeRoy et al (22). Age and sex matched 25 patients with asthma were included in the study as a diseased control group in addition to 16 healthy individuals (HC). All gave written informed consent. The patients who had active infection, history of cancer or less than 18 yrs old were excluded from the study. The study was approved by the ethics committee of the Marmara University (MAR 2009-0040).

Clinical data

In particular, demographic and disease-related information is recorded, including age, gender, ethnicity, smoking status, disease duration since the onset of the first non-Raynaud's symptoms and disease subset (diffuse, limited or sine scleroderma).

Pulmonary function tests (PFT) and lung carbon monoxide transfer factor (TLCO) were evaluated to examine the presence and the severity of pulmonary involvement. The distinction between mild and extensive ILD was made using a FVC value of 70% of the predicted value as a threshold (23). Patients with SSc who were smokers or had other respiratory disorders that could have affected %FVC or %TLCO were excluded from the study. ILD was defined as the presence of typical features such as bibasilar interstitial fibrosis on a HRCT scan of the chest along with restrictive pattern on PFT. Alveolitis-fibrosis index was assessed by a blinded experienced pulmonologist according to "Warrick semiquantitative scoring system" (24-25). Warrick score is composed of severity score, ranging from 0 (normal) to 15 (severe), and extension score, ranging from 0 (normal) to 3 (severe). The two scores are calculated to find total score (ranging from 0 to 30). We also evaluate alveolitis and fibrosis condition by using Warrick score. Ground glass opacities show us alveolitis (ranging from 0 to 4), other parameters (irregular pleural margins, septal/subpleural lines, honeycombing, subpleural cysts) show us fibrosis (ranging from 0 to 26). SSc disease severity scores (26) were recorded as well.

The protocol is approved by the local medical ethics committee.

Determination of Caveolin-1, TGF-β and Endothelin-1

Serum and induced sputum samples were collected at the inception. Inception was defined by the first visit of SSc patient during the study period regardless of disease duration or by a visit matched for sample collection in the controls. Induced sputum collection

was performed using the method described by Efthimiadis et al (27). After the cytospin centrifuge procedures at rpm for 5 minutes, prepared slides were dried in air and were fixed with ethanol and stained with the Giemsa stain for examination. If cell viability was lower than 50% or squamous cell contamination was greater than 20% the sample regarded as of poor quality and the results were excluded. The supernatants of the samples with good quality of cell were collected for ELISA.

Serum samples centrifuged for 15 minutes at 1000g within 30 minutes of collection, then all samples were stored at -20˚C. Cav-1(USCN Life, China), TGF-β (Invitrogen, USA) and ET-1(Biomedica, Austria) levels were measured by ELISA technique using commercial kits following manufactures' recommendations.

Cav-1 was determined in the serum and sputum samples by the sandwich ELISA protocol. Briefly, the microtiter plate has been pre-coated with an antibody specific to 0.5 μg Cav-1. (USCN life, China). Standards and samples (serum or sputum) (100 μL) were added to the well with a biotin-conjugated polyclonal antibody preparation specific for Cav-1. The plate was incubated for 2 hours at 37 C. Then, Avidin conjugated to Horseradish Peroxidase (HRP) (100 μL) was added to each microplate well and incubated for 1 hour at 37 C with shaking, the wells were then washed extensively, and 100 μL tetramethyl benzidine substrate solution (TMB) was added and the blue color was allowed to develop for 30 minutes. The enzyme-substrate reaction was stopped by adding 50 μL sulphiric acid solution and the color change was measured by spectrophotometer at a wavelength of 450 ±2 nm. The concentration of Cav-1 in the serum and sputum was then determinated by comparing the O.D. of the samples to the standard curve.

Statistics

Comparisons between groups were made using non-parametric Kruskall-Wallis and Mann-Whitney U tests. Bonferroni correction was applied for multiple comparisons. Data are presented as mean ± standard deviation (SD). Relationship between two continuous variables was examined by using Spearman correlation test. We evaluated the sensitivity and specificity of sputum Cav-1 with an ROC analysis. A two- sided test with $p<0.05$ was considered statistically significant. The statistical analysis was performed using SPSS software, release 13.0 (SPSS;Chicago; IL).

Results

The mean age was 44.5 ±11.5, 44.9±10.1 and 39.8±10.8 years in SSc, asthma patients and HC, respectively. The mean disease duration was 6.6±6.1 years in SSc patients. The baseline characteristics of the SSc patients are highlighted in table 1.

During the study period, 33(60%) SSc patients were on immunosuppressive therapy (table 1). While 45(81.8%) SSc patients had alveolitis or fibrosis on HRCT, none of the asthma patients had ILD findings on radiologic examination. The mean alveolitis index was 2.79±1.24 and fibrosis index 13.6±7.1, in SSc -ILD.

Caveolin-1,TGF-β and ET-1 levels in serum and sputum samples

Serum and sputum results of the SSc patients and the controls are shown in table 2. Cav-1 levels at the inception were lower in serum samples of both

Table 1. Demographic and clinical characteristics of SSc patients

	SSc patients
Age (years) median(min-max)	48 (25-72)
Gender (F/M) (n)	52/3
Disease subsets n(%)	
Limited SSc	25 (45.4)
Diffuse SSc	29 (52.7)
Sine SSc	1 (1.8)
Disease duration(years) median(min-max)	5 (1-29)
Disease severity score* (mean±SD)	5.4 ± 2.6
Interstitial lung disease n(%)	45 (81.8)
FVC (mean±SD) lt	2.74±0.72
% percent	90.2±19.6
Pulmonary hypertension n(%)	8 (14.5)
Digital ulcer n(%)	22 (40)
Scl 70 antibody positivity n(%)	29 (52.7)
Immunsupressive treatment n(%)	33 (60)
Azathiopurine	23 (41.8)
Cyclophosphamide	5 (9)
Mycophenolate mofetil	5 (9)
ET-1 resep. ant. treatment n(%)	7 (12.7)

*SSc severity scale (21)

Table 2. Serum and sputum Cav-1, TGF-β and ET-1 levels of SSc, asthma patients and healthy controls.

	SSc	Asthma	HC
Serum			
Cav-1(ng/ml)	0.29±0.11	0.27±0.07	0.39±0.10*
TGF-β (pg/ml)	8115±4306	7979±4055	8497±3574
ET-1 (fmol/ml)	3.43±6.05**	0.73±0.53	0.58±0.29
Sputum			
Cav-1 (ng/ml)	0.19±0.04***	0.24±0.07	0.28±0.07
TGF-β (pg/ml)	85.6±85.6	156.8±147.5$^{\delta}$	89.5±83.7
ET-1 (fmol/ml)	0.31±0.14	0.31±0.15	0.25±0.16

ANOVA, p<0.001, Results are presented as mean ± SD
** HC vs. SSc and asthma, ** SSc vs. asthma and HC, *** SSc vs. asthma and HC, $^{\delta}$ asthma vs. SSc and HC*

SSc and asthma patients compared to HC (SSc 0.29±0.11 *ng/ml*, asthma 0.27±0.07 *ng/ml* vs. HC 0.39±0.1 *ng/ml*; p<0.01). Cav-1 was found significantly lower in the sputum of SSc patients compared to both control groups (SSc 0.19±0.04 *ng/ml* vs. asthma 0.24±0.07 *ng/ml*, HC 0.29±0.07 *ng/ml*, p<0.001) (Figure 1). When we analyzed SSc patients separately, we did not find significant differences in the serum Cav-1 levels between diffuse SSc and limited SSc patients (0.38±0.16 *ng/ml* vs. 0.28±0.01 *ng/ml*, p=0.06). Presence of Anti-Scl 70 did not correlate with neither serum nor sputum Cav-1 levels or the extent of ILD judging by their Warrick scores.

Although no difference was observed either in the serum TGF-β or the sputum ET-1 levels among the groups, the mean serum ET-1 measurements was found significantly higher in the SSc patients compared to controls (p<0.001). Sputum TGF-β measurements were significantly higher in the asthma patients compared to SSc patients and HC (p<0.05)(table 2).

Associations with disease subsets

None of the serum and sputum Cav-1, TGF-β, and ET-1 levels correlated with the disease severity score, the presence of pulmonary hypertension, and digital ulcer. Sputum Cav-1 levels were found significantly lower in SSc-ILD compared to both control donors and SSc patients without ILD (0.19±0.04 vs.

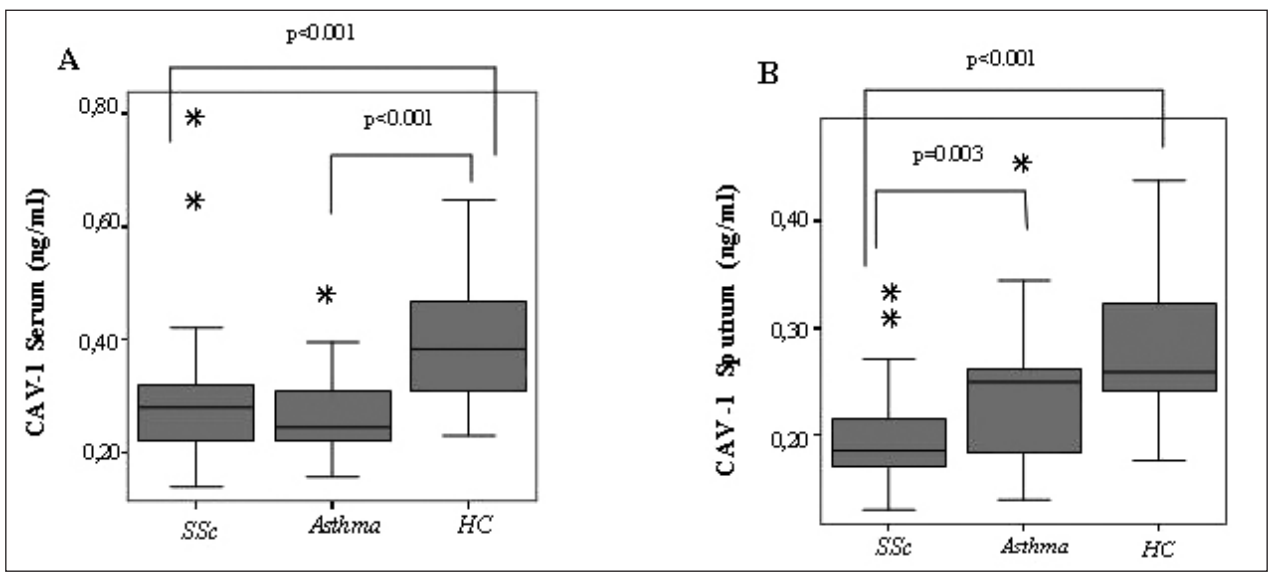

Fig. 1. A) Serum Caveolin-1 levels of the SSc patients and the controls; B) Sputum Caveolin-1 levels of the SSc patients and the controls

0.29 ±0.07 and 0.25±0.07, respectively, p<0.01). At the cut-off value less than 0.19 for sputum Cav-1, the sensitivity and the specificity were 79 % and 61 % for detecting ILD, respectively. The area under the ROC curve for sputum Cav-1 was 0.73. (95% CI, 0.61 to 0.84, p<0.0001) (figure 2).

The mean forced vital capacity (FVC) was significantly lower in SSc-ILD patients (2.57±0.67 L vs. 3.47±0.95 L, SSc-ILD vs. non-ILD, respectively, p<0.02) and the mean alveolitis index was high (2.7±1.2) in the ILD group. In these patients, sputum TGF-β levels correlated positively with the alveolitis index (r= 0.34, p<0.05) and inversely with FVC measurements (r: -0.44, p<0.05). The only association with ET-1 was found between sputum levels and DL-CO/VA (r: 0.33, p<0.03).

Discussion

To the best of our knowledge, this is the first study showing that both the serum and induced sputum Cav-1 levels were found lower in SSc patients.

Evidence linking Cav-1 (the main protein component of caveolae) to a fibrotic phenotype, has been shown in several studies ranging from animal studies to human diseases such as IPF and SSc (12,28-32). De Galdo et al showed decreased Cav-1 in the affected lung and skin biopsies in SSc patients (31). In another study, peripheral blood cells (monocytes and neutrophils) from SSc patients were examined and down regulation of leukocyte Cav-1 was found to be important in lung fibrosis (32). Besides, Cav-1 has been shown as a new susceptibility gene for SSc with an OR: 0.80 in a combined population from French-Italian cohort. (33).

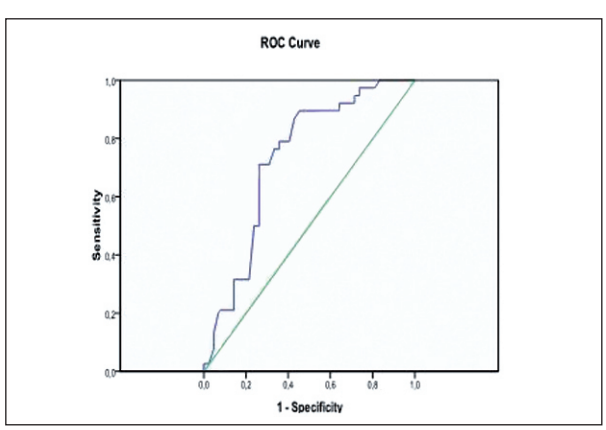

Fig. 2. ROC analysis of sputum Caveolin-1.

Cav-1 plays a crucial role in the regulation of TGF-β signaling due to its participation in receptor internalization. Markedly decreased Cav-1 levels in the affected lungs and skin of SSc patients suggest that these lead an abrogated TGF-β stimulation through the inhibition of Smad 3 activation (30). Therefore, we hypothesized that, examining induced sputum -which reflects bronchoalveolar fluid composition- (20) levels of Cav-1 and TGF-β might help in detecting the progression of pulmonary involvement in patients with SSc. In fact, we did observe significantly lower Cav-1 levels in sputum specimens in SSc-ILD patients.

Despite the fact that our group is rather small, sputum Cav-1 levels display a significant value in detecting lung involvement. However, we did not find any association of the Cav-1 levels with disease activation, disease duration or disease subtype. Our serum Cav-1 results were significantly different than those that were observed in HC. However, we could not demonstrate any difference from those with asthma which is also a disease that could lead to pulmonary fibrosis in severe cases.

Interestingly, we did not observe significant difference in the serum TGF-β levels between the groups. TGF-β is an important molecule that regulates airway remodeling caused by influenced inflammation and connective tissue synthesis (34-37). In one study, down-regulation of Caveolin-1 mRNA levels at lung biopsies and augmentation of TGF-β levels in BAL fluid were shown in acute allergen-induced airway remodeling (34). However, there are some controversies in terms of serum TGF-β levels in SSc patients (38-39). In many studies, increased TGF-β levels were found only in the BAL fluid but not in the serum of SSc (34,40-43). The sputum levels of TGF-β in SSc patients were not different than those that were seen in both control groups. TGF-β also plays a role on airway smooth muscle cells that may explain why we observed increased sputum TGF-β levels in asthma patients but we do not know, what an increased TGF-β implies for the prognosis of asthma. Our study was specifically not designed to address the pathogenetic roles of TGF-β, so all explanations could be speculative in this case. On the other hand, sputum TGF-β levels correlated positively with the alveolitis index and in-

versely with FVC measurements in SSc patients. FVC is a surrogate measure for the severity of lung restriction that has been validated as an outcome measure in randomized trials. These findings may also indirectly support the role of TGF-β in lung fibrosis.

We also tested the serum and sputum ET-1 levels due to its role in the pathogenesis of both fibrotic and vascular manifestations in SSc. Although serum levels were exclusively increased in SSc patients, we did not find any relation with any features of SSc .

We also had some limitations; first, in our cohort more than half of the SSc patients were on immunosuppressives during the study period, therefore we were not able to exclude potential effects of the drugs on the results, however, this is the first study testing Cav-1 as a marker in a cohort of SSc patients, reflecting the real world practice. Second, radiologic evaluation was made by one blinded pneumologist, therefore the lack of interobserver comparisons could decrease the reliability of our Warrick score calculations.

To summarize, we demonstrate that both the serum and sputum Cav-1 levels are lower among SSc patients. However, only decreased sputum Cav-1 levels is associated with ILD in SSc patients, suggesting that Cav-1 plays a role examining the sputum Cav-1 levels may be useful as a non-invasive marker for pathogenesis of SSc- an increased risk in ILD. Further analysis in larger cohorts of SSc patients are needed to definitely rule out define its role as a marker for lung involvement other system associations with Cav-1.

Acknowledgement

This study was funded by Marmara University Research Funds(BAPKO).

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