Association of Psoriasin (S100A7) with Clinical Manifestations of Systemic Sclerosis: Is Its Presence in Whole Saliva a Potential Predictor of Pulmonary Involvement?

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ABSTRACT. ***Objective.*** To evaluate psoriasin (S100A7) expression in whole saliva (WS) of patients with diffuse systemic sclerosis (dSSc) and limited SSc (lSSc), and to correlate its presence with the different clinical manifestations of the disease.

Methods. Forty-four patients with limited or diffuse SSc were enrolled for study. WS proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and psoriasin was identified by Western blot analysis using a specific polyclonal antibody. Patients with other rheumatic diseases with and without lung involvement were enrolled as pathological controls. Statistical analysis was performed to correlate each clinical manifestation with the presence of psoriasin.

Results. Three bands of 12, 24, and 50 kDa corresponding to monomeric and dimeric/multimeric forms of psoriasin were evidenced by immunoblot analysis in WS of 31 out of 44 patients with SSc (70.4%). In the other 13 WS samples, the 12 kDa band was totally absent, while the dimeric and multimeric bands were expressed at optical intensity (OD) levels comparable to the other samples. From a clinical point of view, the presence of 12 kDa monomeric psoriasin was significantly associated with SSc pulmonary involvement and with anti-Scl-70 antibody positivity. No control showed the psoriasin 12 kDa band.

Conclusion. Our results identified salivary 12 kDa psoriasin as a potential predictor of pulmonary involvement in SSc. Thus, a psoriasin assay might be considered as a rapid, noninvasive, useful salivary biomarker for the detection of pulmonary involvement in SSc. (First Release July 15 2008; J Rheumatol 2008;35:1820–4)

Key Indexing Terms:
SALIVA SYSTEMIC SCLEROSIS PSORIASIN
S100A7 PULMONARY INVOLVEMENT

Psoriasin (S100A7) is a small calcium-binding protein of the S100 protein family that was originally identified as an 11.4 kDa protein expressed in psoriatic skin, where it is upregulated[1]. Elevated expression of psoriasin has been demonstrated not only in psoriasis but also in other inflammatory skin diseases, while the biological function of this protein remains unclear[2,3]. In these conditions recombinant human psoriasin was reported as a potent chemotactic inflammatory protein for neutrophils and CD4+ T lymphocytes[4-6]. In addition, a putative role was suggested in invasive malignant lesions of skin such as squamous carcinomas of different organs[7,8], and in a subset of breast tumors[9-12] where psoriasin seemed to participate in tumor progression. Further, a mechanism of antibacterial action in wounds has recently been suggested for psoriasin[13].

In a previous study, using a proteomic approach, we identified psoriasin as one of the specific proteins found in whole saliva (WS) of patients with diffuse systemic sclerosis (dSSc)[14]. Systemic sclerosis (SSc) is a generalized connective tissue disorder, characterized by a wide spectrum of microvascular and immunological abnormalities, leading to progressive accumulation of extracellular matrix components in the skin and visceral organs[15]. Patients with SSc are commonly classified into 2 distinct subsets on the basis of the skin involvement pattern. Diffuse cutaneous SSc is dominated by rapidly progressive fibrosis of the skin, lungs, and internal organs with, in particular, skin thickening involving

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the proximal extremities and/or the trunk[16]. In contrast, limited cutaneous SSc (lSSc) is defined as skin involvement distal to the elbows and knees, with face and neck involvement. The clinical picture of lSSc is dominated by vascular manifestations, while skin and organ fibrosis is generally limited and slow to progress[16,17]. In our previous study, identification of psoriasin was obtained through peptide mass fingerprinting analysis, and the comparison of protein expression between patients with SSc and controls suggested a strong expression of psoriasin only in the patients with SSc compared to healthy subjects[14]. Currently, the clinical implication of psoriasin in SSc is unknown, and therefore worth investigating. Therefore, the aims of our study were: (1) to investigate the presence of psoriasin in WS in a larger number of patients with both diffuse and limited SSc using immunoblot analysis with specific polyclonal antibody; and (2) to correlate the presence of psoriasin with clinical manifestations of SSc by statistical analysis.

MATERIALS AND METHODS

Materials. Thirty percent acrylamide-N,N,N bisacrylamide was acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), ammonium persulfate (APS), N,N,N',N'-tetramethylenediamine (TEMED), and glycine were from MP Biomedical, LLC (Eschwege, Germany). ECL Western Blotting Detection System was from GE Healthcare (Uppsala, Sweden). Goat polyclonal anti-psoriasin (C-15) and donkey anti-goat IgG peroxidase-labeled secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were from standard commercial sources and were of the highest grade available.

Human subjects. Forty-four women with SSc (mean age 55.13 ± 14.75 yrs), 20 with diagnosis of lSSc (mean age 57.7 ± 15.59 yrs), and 24 with dSSc (mean age 53 ± 13.98 yrs), were enrolled. The diagnosis of dSSc and lSSc were made according to the International Classification Criteria for the disease[18]. The clinical features of the patients are summarized in Table 1. Eighteen patients with other rheumatic diseases (Raynaud's phenomenon, rheumatoid arthritis, and polymyositis with lung involvement) were included as pathological controls.

Sample collection and preparation. WS samples were collected early in the morning in standard conditions, i.e., all subjects were asked to have an empty stomach, without having taken any drink or food (including gum or candies) since the night before, as described[19]. Briefly, in order to minimize the degradation of the proteins, the samples were processed immediately and kept on ice during the process. Between 1 and 2.5 ml saliva was obtained from each subject. To remove debris and cells, centrifugation at 14,000 g for 30 min at 4°C was performed and the total protein concentration was determined using Bio-Rad protein assay.

Immunoblot analysis. WS samples were mixed with SDS sample buffer (Laemmly solution) and heated at 100°C for 5 min. Equal amounts of the samples (40 µg of proteins) were loaded onto 15% SDS-PAGE, separated by electrophoresis (Mini vertical gel system, Bio-Rad, Hercules, CA, USA), and electroblotted onto nitrocellulose (0.2 µm). Membranes were blocked in phosphate buffered saline (PBS) (10 mM NaH_2PO_4, pH 7.4, 0.9% NaCl) containing 3% low-fat dried milk and 0.2% Tween 20 (PBS/milk) for 1 h at room temperature (RT) and then incubated overnight in PBS/milk containing primary antibody (goat polyclonal anti-psoriasin antibody, 1:200 dilution) at 4°C followed by 4 washes with PBS/milk at RT. Then, the nitrocellulose was incubated in PBS/milk containing peroxidase-labeled secondary antibody (1:5000 dilution) for 1 h at RT. The washing step was repeated as described above, followed by 2 washes with PBS and 1 with distilled water. The immunoblot was developed using the ECL detection system, scanned (Epson expression 1680 Pro), and the intensity of the immunoreactive bands was quantified by Image J software.

Table 1. Patients' clinical and serological features.

Features	Data
No. of patients	44
Age, yrs	55.13 ± 14.75
Followup, yrs	7.43 ± 7.02
Diffuse SSc	24/44
Limited SSc	20/44
ESR increased	12/44
Hypocomplementemia	8/44
Rodnan skin score (mean ± SD)	9.88 ± 8.00
Telangiectasias	23/44
Melanoderma	27/44
Serological profile	
Scl-70	24/44
ACA	13/44
FAN Hep_2	7/44
Acral ulcers	5/44
History of acral ulcers	18/44
Esophageal involvement	31/44
DLCO reduction	
Mild	20/44
Moderate	16/44
Severe	8/44
Ground-glass opacity (HRCT)	26/44
Lung fibrosis (HRCT)	18/44
Pulmonary hypertension	6/44
Arthralgias	18/44
Arthritis	4/44
Acroosteolysis	6/44
Calcinosis	5/44
Kidney involvement	3/44

SSc: systemic sclerosis; ESR: erythrocyte sedimentation rate; DLCO: carbon monoxide diffusing capacity; HRCT: high resolution computed tomography.

Statistical analysis. To determine the correlations between psoriasin and the clinical and serological measures, statistical analysis was carried out using the chi-squared test. A p value < 0.05 was considered significant. The optical density (OD) values of the immunoreactive bands were expressed as mean ± standard error of the mean (SEM) and the statistical differences were evaluated by unpaired Student t-test. A p value < 0.05 was considered significant. Odds ratio (OR) for categorical variables and Mann-Whitney U-test for continuous variables were investigated.

RESULTS

Psoriasin detection in patients. Western blot analysis of WS in patients with SSc allowed us to identify 2 different patterns of psoriasin expression. In 31 out of 44 (70.4%) patients the psoriasin probing blots revealed 1 intense immunoreactive band of 12 kDa and 2 less intense bands of 24 and 50 kDa. By contrast, in the other 13 WS samples, the 12 kDa band was totally absent, while the dimeric and multimeric bands were expressed at OD levels comparable to the other samples (50 kDa, p = 0.74; 24 kDa, p = 0.38; Figure 1a). In Figure 1c, the molecular weight of the immunoreactive bands and the histograms of the mean ± SEM of OD values of each reactive band are shown for both

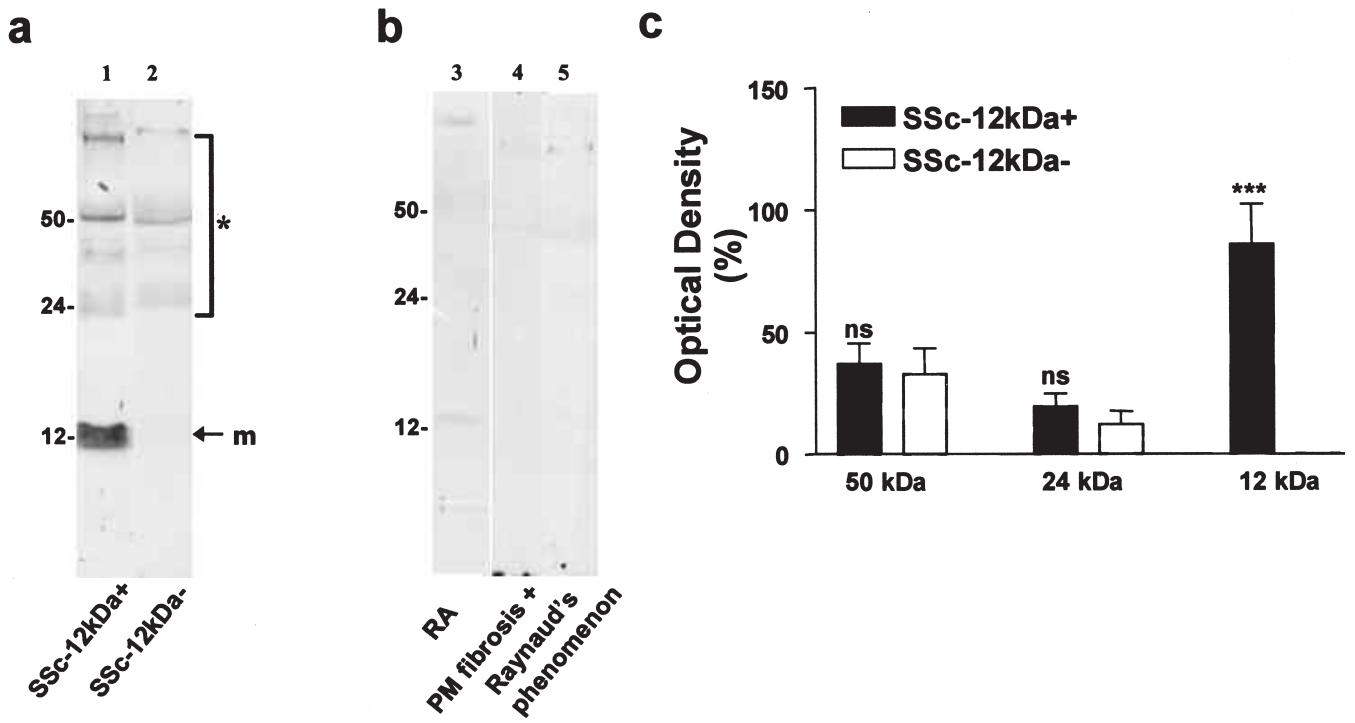

Figure 1. Immunoreactivity patterns of psoriasin antibody to whole saliva sample in patients with systemic sclerosis (SSc) and in pathological controls. (a) Representative psoriasin patterns of SSc-12 kDa psoriasin-positive (lane 1) and 12 kDa psoriasin-negative (lane 2) groups; m: monomeric band; *dimeric/multimeric bands. (b) Representative Western blots of rheumatoid arthritis (RA, lane 3), polymyositis (PM) with pulmonary involvement (lane 4), and Raynaud's phenomenon (lane 5). (c) Quantitative analysis of 12-, 24- and 50-kDa immunoreactive bands. Data for mean ± SEM of optical density values of each reactive band are shown for both the SSc groups. Bars represent the integrated area of 12-, 24-, and 50-kDa bands divided by the integrated area of all bands present in the sample. Significant differences based on t-test (***$p < 0.001$, ns: nonsignificant).

the SSc groups. While the 12 kDa band represents the monomeric form of psoriasin, the higher molecular mass bands might result from a cross-linking process of S100-A7 with itself or with other proteins, as suggested by Ruse, *et al*[20]. In our experiments, the possibility that the multimeric bands represented a covalent complex is supported by their presence even after pretreatment of WS samples both with 50 mM ethylene diamine tetraacetic acid (EDTA) and with 0.1 mM $ZnCl_2$ at RT for 2 h before SDS-PAGE separation (data not shown). None of the pathological controls showed the psoriasin 12 kDa band (Figure 1b).

Psoriasin expression and SSc clinical features. The monomeric 12 kDa psoriasin band was present in patients with both dSSc (17 out of 24) and lSSc (14 out of 20), with no statistical difference ($p = 0.95$; OR 1.04) between the 2 subsets of the disease. From a demographic point of view, the patients showing the 12 kDa monomeric band were significantly older (mean age 58.54 ± 13.64 yrs) than the other group (mean age 47.00 ± 14.58 yrs). Otherwise, no statistical correlation was found between the expression of the 12 kDa band and the latency of the disease ($p = 0.07$). Table 2 gives the most significant clinical and serological correlations between psoriasin and SSc manifestations that were detected using statistical analysis.

Table 2. Statistical correlations between psoriasin and SSc clinical and serological features.

Clinical Features (vs psoriasin)	p
Lung fibrosis, HRCT	0.02
Ground-glass opacity, HRCT	0.0005
Moderate-severe DLCO decreased	0.0007
Anti-Scl-70 antibodies	0.02
Pulmonary hypertension	0.92
Increased ESR	0.06
Hypocomplementemia	0.75
Current acral ulcers	0.12
History of acral ulcers	0.83
Telangiectasias	0.23
Melanoderma	0.48
Esophageal involvement	0.40
Arthralgias	0.25
Arthritis	0.83
Capillaroscopic pattern late	0.71
Acroosteolysis	0.82
Kidney involvement	0.24
Calcinosis	0.12
Latency of disease	0.7
Rodnan skin score	0.08

SSc: systemic sclerosis; ESR: erythrocyte sedimentation rate; DLCO: carbon monoxide diffusing capacity; HRCT: high resolution computed tomography; $p < 0.05$ was considered significant.

Presence of psoriasin seems to correlate positively with lung involvement, and in particular with a moderate-severe decrease of carbon monoxide diffusing capacity (DLCO) ($p = 0.0007$; OR 8.94) and with the evidence of ground-glass opacity on high resolution computed tomography (HRCT) ($p = 0.0005$; OR 10.25) and pulmonary fibrosis ($p = 0.02$; OR 4.58). Moreover, a positive correlation was detected between psoriasin 12 kDa band expression and anti-Scl-70 antibodies ($p = 0.02$; OR 3.78). By contrast, no statistically significant correlations were found with the Rodnan skin score, the other skin involvement manifestations (telangiectasias, melanoderma, ulcers), or with esophageal symptoms, kidney impairment, forced expiratory vital capacity and pulmonary hypertension.

DISCUSSION

We have confirmed using immunoblot analysis our previous observation of a strong expression of 12 kDa psoriasin band in WS in a larger number of patients with SSc and the absence of this protein in other pathological conditions. Our study was performed to investigate the potential relevance and clinical correlations between the monomeric psoriasin expression and a broad spectrum of clinical and serological features of SSc including skin and visceral disease manifestations. Our results have outlined a significant correlation between psoriasin and lung involvement in SSc. In particular, we found a highly significant correlation between psoriasin and a moderate-severe decrease of DLCO, as well as ground-glass opacity and pulmonary fibrosis on HRCT. According to the literature[21-23], lung involvement in SSc has been reported in up to 80–90% of patients and, in particular, it is widely accepted that pulmonary fibrosis is associated with the presence of anti-Scl-70 antibodies[24-28]. Interestingly, our data confirmed a clear correlation between psoriasin expression and the presence of anti-Scl-70 antibodies ($p = 0.02$), reinforcing the link between psoriasin-lung involvement and anti-Scl-70 antibodies. It is noteworthy that the absence of the psoriasin 12 kDa band in the other pathological controls, including patients with other connective tissue diseases with and without pulmonary involvement and patients with Raynaud's phenomenon, seems to sustain the relevance of the expression of psoriasin in SSc and its peculiar correlation with lung involvement in this disease.

In the last few years, several markers have been correlated with pulmonary fibrosis in scleroderma including monocyte chemoattractant protein-1[29], macrophage inflammatory protein-1α[29], connective tissue growth factor[30], soluble interleukin 6 (IL-6) receptor levels[31], tissue inhibitor of metalloproteinase 2[32], and surfactant D[33]. Moreover, recent studies[34,35] have focused attention on a glycoprotein antigen expressed mainly on type II pneumocytes in alveoli and respiratory bronchiolar epithelial cells, and have shown that serum keratin 6L (KL-6) levels are associated with new onset or deterioration of pulmonary fibrosis in SSc, directly reflecting alveolar damage and inflammation[35]. Finally, the analysis of bronchoalveolar lavage fluid proteome from systemic patients with lung fibrosis[36] has shown a significant upregulation of calgranulin B. The latter is an S100 protein that could be involved in mechanisms that drive lung fibrogenesis through several pathways (i.e., extravasation of neutrophils, production of IL-8, fibroblast proliferation, and endothelial activation)[37-39]. In this context, psoriasin, as a member of the same S100 protein family, arises as a new, not yet identified, potential salivary marker of SSc associated with pulmonary involvement. The pathogenetic relationship between psoriasin and lung involvement is still far from being completely clarified. It could be hypothesized that the link between psoriasin and lung involvement may be represented by the protein chemotactic action for immune cells. This role of psoriasin as a chemoattractant agent, able to stimulate the neutrophil and CD4+ T lymphocyte infiltration, has already been demonstrated in psoriasis epidermis[1,5,6] and in other skin inflammatory conditions[40]. From a different point of view, psoriasin expression could also be related to the injury of alveolar squamous epithelial cells, which is recognized as one of the initial steps of pulmonary fibrosis. Intriguingly, psoriasin, as an induced protein of squamous epithelial cells, is well documented in squamous cell tumorigenesis[6,9,12].

The diagnostic power of psoriasin has been analyzed in comparison to anti-Scl-70, pulmonary function testing, and HRCT (Table 3). Despite a rather moderate specificity (50%), the psoriasin test appears to be highly sensitive (85%) and, from this point of view, it seems to be useful to correctly identify the presence of pulmonary involvement in SSc. This high sensitivity is crucial for early diagnosis and treatment of pathological conditions and particularly for detecting the presence of pulmonary involvement in SSc. Then, even if further studies are needed to confirm our observation, the introduction of a simple, easy, noninvasive screening test might represent another valid tool for the management of patients with SSc. Moreover, it is desirable that, in the near future, a reproducible and less expensive ELISA test, specifically oriented to the identification of psoriasin in WS, could be available for use in a clinical setting.

Table 3. Diagnostic power of psoriasin.

	PPV	NPV	Sensitivity	Specificity
Psoriasin	0.71	0.70	0.85	0.50
Anti-Scl-70	0.71	0.55	0.65	0.61
DLCO	0.96	0.85	0.88	0.94
PFT	0.86	0.44	0.24	0.94
HRCT	0.89	0.87	0.92	0.82

DLCO: carbon monoxide diffusing capacity; PFT: pulmonary function test; HRCT: high resolution computed tomography; PPV: positive predictive value; NPV: negative predictive value.

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