Increased messenger RNA levels of the mesenchymal cadherin-11 in the peripheral blood of systemic sclerosis patients correlate with diffuse skin involvement

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ABSTRACT

Objective. *Cadherin-11 is a cell-cell adhesion molecule also involved in cellular migration and invasion. Experimental studies implicated this molecule in inflammatory arthritis and fibrosing conditions. Moreover, cadherin-11 protein is hyper-expressed on fibroblasts and macrophages in the skin of systemic sclerosis (SSc) patients, whereas the respective mRNA levels correlate with skin thickness. Herein, we searched for possible cadherin-11 expression also in cells that circulate in SSc peripheral blood.*

Methods. *Cadherin-11 mRNA was quantified by real-time reverse transcription-polymerase chain reaction in 3 ml blood samples obtained from 71 SSc patients (aged 53±2 years, 65 women) and 35 control non-SSc patients with Raynaud's phenomenon.*

Results. *Cadherin-11 mRNA transcripts were detected in blood samples from 39% of patients with diffuse SSc, versus 16% of those with limited SSc, versus 6% and 16% of patients with idiopathic or associated with other connective tissue diseases Raynaud's phenomenon, respectively (p=0.049). Cadherin-11 mRNA levels in SSc patients were increased by 3.74-fold comparing to controls (p=0.036). By multivariate logistic regression analysis we found that diffuse skin involvement correlated, independently of age, gender, disease duration, lung involvement, digital ulcers, inflammatory indices or anti-Scl-70 autoantibody presence, with cadherin-11 mRNA positivity (p=0.028), but also with increased cadherin-11 mRNA levels (≥3-fold of non-SSc levels, p=0.011).*

Conclusion. *Cadherin-11 may be hyper-expressed in the peripheral blood of diffuse SSc patients. Studies on the origin and possible pathogenic function of these circulating cells may shed light into the complex disease pathogenesis and further support the notion that cadherin-11 is a potential therapeutic target in SSc.*

Introduction

Systemic sclerosis (SSc) is a progressive autoimmune systemic disease characterised by vasculopathy and tissue fibrosis. Recent therapeutic approaches aiming at preventing vascular complications have shown considerable success, while novel candidate molecules implicated in the pathogenesis of SSc-related vasculopathy have been lately identified, including among others the angiogenic Epidermal Growth Factor-Like domain 7 and adhesion molecules of the junctional adhesion molecule family (1). However, SSc is still associated with highly significant morbidity and mortality since all strategies to reduce or directly control fibrosis have proven inefficient (2). Progressive fibrosis of the skin, in particular of the hands and face, imparts significant disability and impairs health related quality of life (3), while internal organ involvement often leads to life threatening complications. Recent advances in understanding the molecular mechanisms driving the disease and the profibrotic roles of TGF-beta and tyrosine kinases led to clinical trials, but failed to translate into effective treatment approaches (4, 5).

One of the most recent candidate therapeutic targets in SSc is the mesenchymal cadherin-11, also known as osteoblast cadherin, which is primarily expressed on fibroblasts (6). This molecule mediates cell-cell adhesion and is also involved in cellular migration, tumor invasion and metastasis, as well as in inflammatory arthritis (7). In addition,

a pathogenetic role of cadherin-11 in fibrosing conditions has been suggested by elegant experimental studies. Using the bleomycin model of pulmonary fibrosis, Shneider *et al*. showed that cadherin-11 expression was upregulated on fibroblasts, epithelial cells and alveolar macrophages, whereas decreased bleomycin-induced fibrotic endpoints were noted in cadherin-11 deficient mice compared to wild-type mice. Furthermore, anti-cadherin-11 neutralising monoclonal antibodies successfully treated established pulmonary fibrosis induced by bleomycin (8). The same group of investigators showed that relative to wild-type mice, cadherin-11 deficient mice had also markedly decreased dermal fibrosis when injected with bleomycin, as evaluated by skin thickness, collagen levels, myofibroblasts accumulation and profibrotic gene expression in the lesional skin. More importantly, anti-cadherin-11 therapy decreased dermal fibrosis at various time points in this fibrosis model (9).

At the clinical level, two independent microarray studies identified increased cadherin-11 transcripts in the affected skin of patients with SSc compared to healthy-control derived skin (10, 11). Moreover, hyper-expression of cadherin-11 was observed by immunohistochemistry in fibroblasts, epithelial cells, and alveolar macrophages of patients with pulmonary fibrosis (8), as well as in the affected skin of SSc patients, where cadherin-11 expression was localised to dermal fibroblasts and resident macrophages (9). The respective cadherin-11 mRNA levels were increased and correlated with the modified Rodnan skin score in these patients (9).

In the present study we examined whether increased cadherin-11 expression can also be identified in cells that circulate in the peripheral blood of SSc patients. Indeed, cadherin-11 mRNA transcripts, reflecting the presence of viable cadherin-11 expressing cells, were more frequently and at higher levels found in patients with SSc than non-SSc control patients with Raynaud's phenomenon and correlated independently with the presence of diffuse skin involvement.

Table I. Demographic, clinical and laboratory characteristics of SSc patients and control patients.

	Diffuse SSc	Limited SSc	Raynaud's phenomenon, idiopathic	Raynaud's phenomenon associated with other CTD
n.	28	43	16	19
Age (mean±SD)	49±14 years	55±13 years	37±16 years	49±15 years
Female sex n. (%)	23 (82%)	42 (98%)	15 (94%)	15 (79%)
Disease duration (mean±SD)	8.0±5.3 years	8.1±8.3 years	7.2±12.3 years	8.6±11.1 years
mRSS (mean±SD)	15.0±10.6	3.8±3.3	-	-
Pulmonary fibrosis present n. (%)	23 (82%)	21 (49%)	-	-
RVSP ≥45mmHg n. (%)	4/25 (16%)	2/35 (6%)	-	-
FVC (mean±SD)	80.6±17.7	93.4±15.5	-	-
DLCO (mean±SD)	58.2±17.4	68.2±19.3	-	-
ESR (mean±SD)	37.7±18.9 mm/hr	40.3±24.7 mm/hr	25.9±31.2 mm/hr	33.0±20.7 mm/hr
Digital ulcers ever during disease course n. (%)	19 (68%)	15 (35%)	0	4 (21%)
Anti-Scl 70 positive n. (%)	21 (75%)	21 (49%)	-	-
Capillaroscopy pattern				
normal n. (%)	0	3 (7%)	9 (56%)	11 (58%)
early n. (%)	2 (8%)	11 (27%)	7 (44%)	7 (37%)
active n. (%)	12 (46%)	22 (54%)	0	1 (5%)
late n. (%)	12 (46%)	5 (12%)	0	0
Cadherin-11 mRNA positivity	11 (39%)*	7 (16%)	1 (6%)	3 (16%)

*Fisher's exact test: p=0.049.

Patients and methods

SSc patient- and control-derived peripheral blood samples

Three ml of peripheral blood was obtained from consecutive patients with SSc fulfilling the 2013 diagnostic criteria (12), after discarding the first 3 ml to avoid dermal fibroblast contamination. Consecutive patients who underwent capillaroscopy for Raynaud's phenomenon, either idiopathic or associated with systemic lupus erythematosus, mixed connective tissue disease, or rheumatoid arthritis served as controls. Detailed clinical examination, erythrocyte sedimentation rate (ESR) and anti-Scl-70 measurements were performed and the modified Rodnan skin score in SSc patients was calculated. Patients and controls with active synovitis at the time of sampling, as defined by the presence of at least one simultaneously swollen and tender joint (13) were excluded. Clinical and demographic characteristics of all study participants are shown in Table I. The study was approved by the Laikon Hopsital ethics committee and all subjects provided written informed consent according to the Declaration of Helsinki.

Determination of cadherin-11 mRNA levels by Real-time QPCR

Peripheral blood samples were subjected to erythrocyte lysis and 1 μg of each total extracted RNA (RT-118; MRC Inc. Cincinnati, OH, USA) was reverse transcribed to cDNA and quantified by QRT-PCR using specific primers for Cdh-11 as previously described in detail (13). All samples were run in duplicate and the final values were averaged. Relative differences in cadherin-11 mRNA levels were estimated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and B-actin as the endogenous control.

Statistical analysis

Chi square test with Yate's correction, Fisher's exact test, unpaired Student's *t*-test, as well as multivariate logistic regression analysis, including demographic, clinical and laboratory factors that could affect cadherin-11 positivity in QRT-PCR, were used as appropriate; $p<0.05$ was considered significant.

Results

Based on the findings described in our previous work (13), showing that the quantitative real-time RT-PCR em-

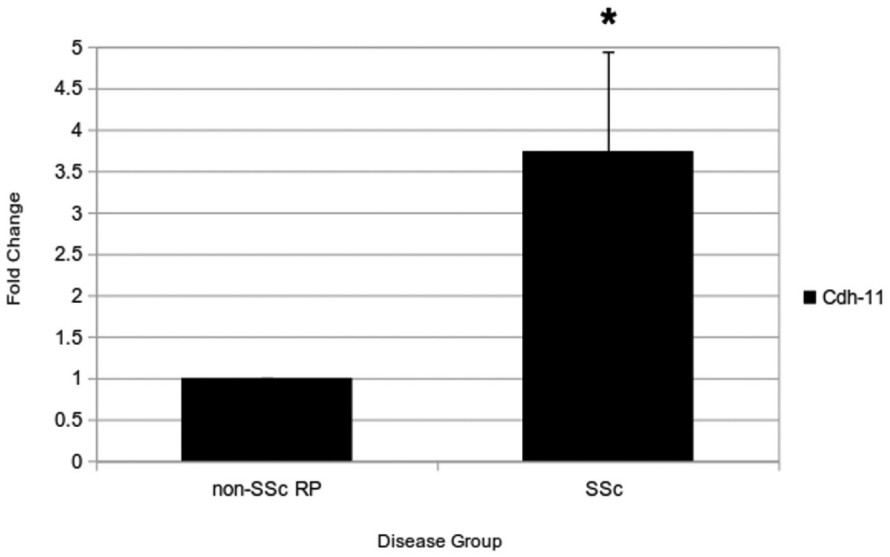

Fig. 1. Circulating mRNA levels of cadherin-11 in systemic-sclerosis patients and non-systemic sclerosis control patients with Raynauld's phenomenon. Following melting curve analysis the relative differences in cadherin-11 mRNA levels between SSc and non-SSc Raynaud's phenomenon patients were estimated as fold changes, using B-actin as the endogenous control and the average DCt value of non-SSc Raynaud's phenomenon patients as the calibrator sample (fold 1). Results indicate a significant upregulation by 3.74-fold of cadherin-11 mRNA levels (p=0.036) in SSc. Each sample was analysed in duplicate and the final value was averaged, whereas Ct values above 36.4 were excluded from quantitative analysis.
Ct: threshold cycle; qPCR: quantitative real time polymerase chain reaction.

Table II. Summary of logistic regression analysis for variables* predicting cadherin-11 mRNA positivity within systemic sclerosis patients.

Predictor	β	SE β	p-value	e^{β} (odds ratio)	95% Confidence interval for e^{β}
Constant	-2.753	1.806	0.127	not applicable	not applicable
Disease subtype	**1.598**	**0.727**	**0.028**	**4.945**	**1.190–20.550**
Pulmonary fibrosis	0.021	0.769	0.978	1.021	0.226–4.608
Disease duration	0.055	0.043	0.203	1.057	0.971–1.150
Gender	-1.155	1.211	0.340	0.315	0.029–3.381
Age	0.005	0.027	0.868	1.005	0.952–1.060
Digital ulcers	-0.719	0.675	0.287	0.487	0.130–1.831
ESR	0.006	0.015	0.686	1.006	0.977–1.036
Anti-Scl 70	0.749	0.800	0.349	2.114	0.441–10.144

*Disease subtype coded as 1 for dcSSc and 0 for lcSSc. Pulmonary fibrosis, presence of anti-Scl 70 antibody and digital ulcers coded as 1 for yes and 0 for no. Gender coded as 1 for male and 0 for female.

ployed herein is able to detect at least 10 cadherin-11 expressing cells per ml of peripheral blood, a total of 106 samples derived from SSc and non-SSc control patients with Raynaud's phenomenon was examined (Table I).

Cadherin-11 mRNA transcripts were detected in 39% of patients with diffuse *versus* 16% of patients with limited SSc, *versus* 6% of patients with idiopathic Raynaud's phenomenon, *versus* 16% of patients with other connective tissue diseases and Raynaud's phenomenon (p=0.049). Interestingly, cadherin-11 mRNA levels in the peripheral blood of positive SSc patients were significantly (p=0.036) increased by 3.74-fold comparing to positive non-SSc control subjects with Raynaud's phenomenon (Fig. 1).

Comparison of various demographic, clinical and laboratory parameters between cadherin-11 mRNA positive and negative SSc patients showed no difference in mean age (53.22±14.4 *vs*. 52.4±13.2 years), female sex (94% *vs*. 91%), disease duration (9.81±8.0 *vs*. 7.44±6.9 years), anti Scl-70 antibody positivity (72% *vs*. 55%), mRSS (10.29±10.4 *vs*. 7.57±8.4), presence of pulmonary fibrosis (72% *vs*. 31%), forced vital capacity (84.88±18.4 *vs*. 88.93±17.35), DLCO (61.56±18.17 *vs*. 65.06±19.53), digital ulcers (44% *vs*. 51%), erythrocyte sedimentation rate (40.78±22.15 *vs*. 38.80±22.8 mm/hr), capillaroscopic pattern and the presence of right ventricular systolic pressure above 45 mmHg in cardiac echocardiography (6% *vs*. 12%). In contrast, cadherin-11 mRNA positivity in patients with SSc was associated with the presence of diffuse skin involvement (61% in positive *vs*. 32% in negative patients, p=0.049, chi-square test).

Furthermore, by multivariate logistic regression analysis, cadherin-11 positivity correlated significantly with diffuse skin involvement, independently of age, gender, disease duration, lung involvement, digital ulcers, inflammatory indices or anti-Scl-70 autoantibody presence (p=0.028, Table II). A similar independent correlation (p=0.011) between diffuse skin involvement and higher individual levels of cadherin-11 mRNA (arbitrarily considered as ≥3-fold of average levels in non-SSc patients) was also observed.

Discussion

Several lines of experimental evidence have suggested a role of cadherin-11 in the pathogenesis of synovial inflammation and tissue fibrosis (6-9). We have previously shown that cadherin-11 mRNA transcripts are found 4 times more frequently in the peripheral blood of patients with moderately/severely active RA than healthy blood donors, using the same method applied herein. False-positive results could have arisen as a consequence of introduction in the circulation of skin fibroblasts during blood sampling, but the highly significant difference clearly suggested an association between the presence of cadherin-11 expressing cells in the blood and clinically active synovitis in patients with RA (13). In the present study a possible contamination of the obtained blood sample with dermal fibroblasts was minimised by discarding the first 3 ml of blood after venipuncture. Also, by excluding all patients with arthritis at the time of sampling a positive result that could be associated

with active synovitis was avoided. We studied a relatively large group of SSc, which is representative of the general SSc population followed in our centre (14) and found that cadherin-11 is clearly upregulated in their peripheral blood compared to non-SSc patients with Raynaud's phenomenon (Fig. 1). As confirmed by multivariate logistic regression analysis both the detection (positivity) and increased levels of cadherin-11 mRNA transcripts in the peripheral blood of individual SSc patients were independent of age, gender, disease duration, interstitial lung disease, digital ulcers, ESR and the presence of anti-Scl-70 antibodies, but correlated significantly with the presence of the diffuse disease form.

The current experiments have not addressed the nature of circulating cadherin-11 expressing cells in SSc patients. Given the fact that cadherin-11 can be expressed on bone marrow-derived cells, such as alveolar macrophages in patients with pulmonary fibrosis (8) and resident macrophages found in the skin of SSc patients (9), we believe that at least a portion of these cells should belong to the monocyte lineage. Notably, in patients with active RA the presence of cadherin-11 expressing cells in synovial fluid and peripheral blood has< been verified by double-stain flow cytometry using the pan-haematopoietic marker CD45, and their estimated number varied between 100 and more than 1000 cells/ml of synovial fluid *versus* 10-50 cells/ml of peripheral blood (13). However, an average of 60% cadherin-11 positive cells found in peripheral blood of 6 out of 12 RA patients lacked CD45 expression, implying the possibility of circulating synovial fibroblasts. Such rare cells could hypothetically enter the circulation as the synovium is being transformed into a hyperplastic, invasive tissue with new vessel formation (13). Along this line, further studies are warranted in order to verify whether cadherin-11 expressing cells lacking CD45 expression, that may possibly represent fibroblasts, exist in systemic circulation of SSc patients. Such cells could possibly be detected and visualised in SSc peripheral blood by the fluid phase biopsy assay. This method has been successfully employed for demonstration of circulating tumor cells in patients with metastatic cancers (15), as well as of circulating endothelial cells after myocardial infarction (16).

To conclude, cell-cell interactions mediated by cadherin-11 may have a pathogenetic role in SSc since the presence of cadherin-11-expressing cells in the peripheral blood of these patients seems to associate with diffuse skin involvement. Further studies on the origin and possible pathogenic function of such circulating cells may shed light into the complex pathogenesis of the disease and may identify that cadherin-11 is a legitimate therapeutic target in SSc.

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