

Circulating miR-142-3p levels in patients with systemic sclerosis

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Summary

Background. Recently, increased evidence has shown that serum micro (mi)RNA levels are a useful biomarker for the diagnosis, prognosis and therapeutic value of various diseases. However, serum miRNA has not been investigated in patients with systemic sclerosis (SSc), to our knowledge.

Aim. To investigate the possibility that serum levels of *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p), one of the miRNAs regulating the expression of integrin αV , could be a specific disease marker for SSc.

Methods. Serum samples were obtained from 61 patients with SSc and 20 healthy controls. Patients with systemic lupus erythematosus (SLE), dermatomyositis (DM) and scleroderma spectrum disorder (SSD), who did not fulfil American College of Rheumatology criteria for SSc but might develop SSc in the future, were included as disease controls in this study. miRNAs were purified from serum, and miR-142-3p levels were measured with a quantitative real-time PCR assay.

Results. Serum miR-142-3p levels in patients with SSc were significantly higher than in patients with SSD, SLE or DM, and healthy control groups. Patients with increased miR-142-3p levels tended to have a short sublingual frenulum.

Conclusions. Our data indicate that serum levels of miR-142-3p may be elevated specifically in patients with SSc, correlating with the severity of this disease, and may be useful diagnostic markers for the presence of SSc and for the differentiation of SSc from SSD.

Introduction

Systemic sclerosis (SSc) or scleroderma is an acquired disorder that typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, auto-immune attack and vascular damage, leading to the activation of fibroblasts and disturbed interactions with different components of the extracellular matrix (ECM).^{1,2} Thus, abnormal SSc fibroblasts, which are responsible for the fibrosis, may develop from a subset

of cells that have escaped from normal control mechanisms.^{3,4}

Although the mechanism of activation of dermal fibroblasts in SSc is presently unknown, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by transforming growth factor (TGF)- $\beta 1$.⁵ The principal effect of TGF- $\beta 1$ on mesenchymal cells is its stimulation of ECM deposition. Fibroblasts from affected SSc skin cultured *in vitro* produce excessive amounts of various collagens, mainly types I and III,^{6,7} and display increased transcription of corresponding genes,⁸ suggesting that the activation of dermal fibroblasts in SSc may be a result of stimulation by TGF- $\beta 1$ signalling.

TGF- $\beta 1$ is normally secreted as a latent precursor complex, including the bioactive peptide of TGF- $\beta 1$ and latency-associated peptide (LAP)- $\beta 1$. TGF- $\beta 1$ forms a noncovalent complex with LAP- $\beta 1$, called the small

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latent complex (SLC). In this configuration, TGF- β 1 is inactive because it cannot bind to its receptors. TGF- β 1 activation is a complex process, involving conformational changes induced by either cleavage of LAP- β 1 by various proteases such as plasmin, or by physical interactions of LAP- β 1 with other proteins such as integrins and thrombospondin1, leading to the release of bioactive and mature TGF- β 1.^{9–12}

We previously reported that total (active plus latent) and active TGF- β 1 levels in culture media are as high for SSc fibroblasts as for normal fibroblasts.¹³ Thus, the activation of dermal fibroblasts in SSc may be a result of stimulation by autocrine TGF- β signalling, without increasing the concentration of active TGF- β 1. To explain how the endogenous latent TGF- β 1 is activated in SSc fibroblasts, we recently reported overexpression of α V β 5 and α V β 3 integrin in these cells. These integrins may recruit and activate the SLC on the cell surface of SSc fibroblasts. Recruitment and/or activation of latent TGF- β 1 in the pericellular region may enhance the incidence of interaction between active TGF- β 1 and its receptors, leading to the self-activation of SSc fibroblasts without increasing levels of TGF- β 1.^{11,14} Therefore, integrin overexpression is thought to be the most upstream event of TGF- β 1 activation and collagen upregulation in SSc fibroblasts. However, the mechanism of integrin overexpression in SSc is still unknown.

In the present study, we focused on micro (mi)RNA as the regulator of integrin expression. miRNAs, short ribonucleic acid molecules, on average only 22 nucleotides long, are post-transcriptional regulators that bind to complementary sequences in the 3' untranslated regions (UTRs) of mRNAs, leading to gene silencing. We investigated serum levels of *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p) in patients with SSc. miR-142-3p can bind to the 3' UTR of the integrin α V subunit, based on miRNA target gene predictions using TargetScan (version 5.1; <http://www.targetscan.org/>), a leading software program in the field.¹⁵ Recently, there has been increasing evidence showing that serum miRNA levels are a useful biomarker for diagnosis, prognosis and therapeutic value, especially for various malignant tumours. Thus, we investigated the possibility that serum levels of miR-142-3p might be a disease marker for SSc, reflecting the overexpression of integrin α V levels.

Methods

The study was carried out in accordance with the Declaration of Helsinki. Institutional review board

approval and written informed consent were obtained before participants were entered into the study.

Clinical assessment and patient material

Serum samples were obtained from 61 patients with SSc (12 men, 49 women; mean \pm SD age 60.4 \pm 13.2, range 29–85). All patients with SSc were grouped according to the classification system proposed by LeRoy *et al.*¹⁶ Based on previous descriptions,¹⁷ 23 patients had diffuse cutaneous (dc)SSc and 38 patients had limited cutaneous (lc)SSc. In addition, 12 patients diagnosed as having scleroderma spectrum disorder (SSD; 1 man, 11 women; mean \pm SD age 55.4 \pm 11.9, range 44–80), who did not fulfil the American College of Rheumatology criteria for SSc but might go on to develop SSc in the future based on the criteria proposed by Ihn *et al.*, were also included.^{18–20}

Control serum samples were also collected from 20 healthy volunteers matched for age and gender (6 men, 14 women; mean \pm SD age 44.0 \pm 13.8, range 28–83). We also included serum samples from other connective-tissue disorders as disease controls; eight patients with systemic lupus erythematosus (SLE; two men, six women; mean \pm SD age 38.9 \pm 15.3, range 22–65) and eight patients with dermatomyositis (DM; three men, five women; mean \pm SD age 64.9 \pm 17.6, range 31–86).

Diagnostic method of SSD using the points system

For the diagnosis of SSD, a total score was obtained as the sum of the following five factors:^{18–20} (i) extent of skin sclerosis: 10 points for truncal sclerosis, 5 points for skin sclerosis limited to the extremities and face, 3 points for sclerodactylia alone and 1 point for swollen fingers; (ii) pulmonary changes: 4 points for pulmonary fibrosis accompanied by decreased vital capacity (\leq 80%) and 2 points for pulmonary fibrosis accompanied by normal vital capacity (\geq 80%); (iii) antinuclear antibody (ANA): 5 points for positive antitopoisomerase I antibody, 3 points for positive anticentromere antibody or anti-U1 ribonucleoprotein antibody, 2 points for antinucleolar antibody and 1 point for other positive ANA; (iv) pattern of Raynaud phenomenon: 3 points for triphasic (pale purple-red), 2 points for biphasic (two of the above colours) and bilateral, and 1 point for biphasic and hemilateral, or monophasic (pale or purple only) and bilateral; and (v) nailfold bleeding (NFB): 2 points for NFB in \geq 3 fingers, and 1 point for NFB in one or two fingers.

Conditions with \geq 9 are consistent with SSc, and those with 5–8 points consistent with SSD.

Extraction of microRNA and quantitative real-time PCR

There has been no report demonstrating the expression of hsa-miR-142-3p in cell-free body fluid, to our knowledge. To validate that the miRNA is indeed detectable in human serum, miRNA was extracted from sera of healthy individuals, and the levels of miR-142-3p were determined by quantitative real-time PCR using a primer set specific for miR-142-3p.

Isolation of miRNA from serum samples was performed with a commercial kit (miRNeasy RNA Isolation kit; Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions with minor modification.²¹ Briefly, 100 µL of serum were supplemented with 5 µL of 5 fmol/µL synthetic non-human miRNA [*Caenorhabditis elegans* miR-54 (cel-miR-54), Takara Bio Inc., Shiga, Japan] as controls, providing an internal reference for normalization of technical variations between samples. Lysis reagent (1 mL; Qiazol solution; Qiagen Inc.) was added and mixed well by vortex, then samples were incubated at room temperature for 5 min. The aqueous and organic phases were separated by the addition of chloroform. The aqueous phase was then added to columns (RNeasy and RNeasy MinElute spin columns; Qiagen Inc.), and miRNA eluted with nuclease-free water.

Complementary (c)DNA was synthesized from miRNA (Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR kit; Takara Bio Inc). Quantitative real-time PCR was carried out on a thermal cycler (Thermal Cycler Dice (TP800)[®]; Takara Inc.), and primers and templates were mixed with the SYBR Premix. The sequence of the hsa-miR-142-3p primer was designed based on the miRBase sequence (<http://www.mirbase.org>): TGT-AGTGTTCCTACTTTATGGA. DNA was amplified using 50 cycles of denaturation for 5 s at 95 °C and annealing for 20 s at 60 °C. The relative fold changes of gene expression between hsa-miR-142-3p and cel-miR-54 were calculated using standard curves.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test for the comparison of serum miR-142-3p levels, the Student *t*-test for the comparison of means in clinical and serological features, and the Fisher exact probability test for the analysis of frequency in clinical and serological features. The Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and Statcel 97 (OMS, Tokorozawa, Japan) were used to evaluate statistical significance. $P < 0.05$ was considered significant.

Results

hsa-miR-142-3p was present in the serum

We found detectable and quantitative levels of hsa-miR-142-3p in the serum of healthy controls (Fig. 1). Amplification of hsa-miR-142-3p was observed, and Ct values were increased by serial dilution of the miRNA.

Serum concentrations of hsa-miR-142-3p

Serum miR-142-3p levels in patients with SSc are shown in Fig. 2. Serum miR-142-3p levels in patients with SSc were significantly higher than those in healthy control subjects or in patients with SSD, SLE or DM. When patients with SSc were classified as lcSSc or dcSSc as described in Methods, there was no significant difference between the two groups. In addition, there was no significant difference between patients with SSD and healthy controls. When the cut-off value was set as the mean + 2SD of the healthy controls, the values for all patients with SSD were below the cut-off line, whereas increased serum concentrations of miR-142-3p were still present in 32 of the 61 patients (52.5%) with SSc.

Correlation of serum miR-142-3p levels with clinical and laboratory results for patients with SSc

Table 1 shows the association of serum 142-3p levels with the clinical features in patients with SSc. Although there was no significant difference ($P = 0.08$), patients

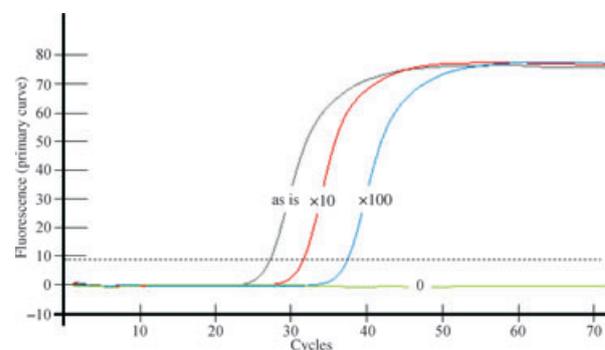


Figure 1 *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p) was found in serum samples from healthy controls. Serial dilutions of cDNA (10-fold dilution, 100-fold dilution and undiluted) synthesized from serum micro RNA were used as template for real-time PCR. Amplification curves of gene-specific transcripts are shown to illustrate the process of exponential increase of fluorescence. The horizontal dotted line indicates the threshold.

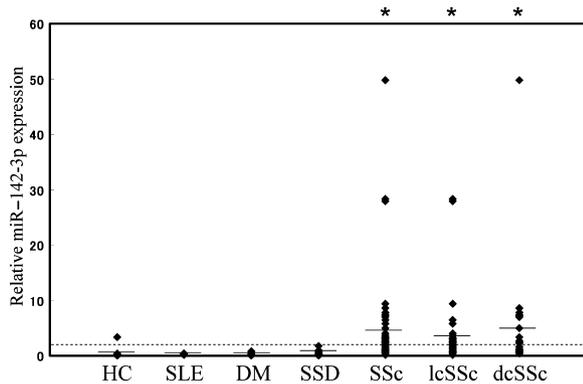


Figure 2 Serum concentrations of *Homo sapiens* miR-142 stem-loop (hsa-miR-142-3p) in patients with systemic sclerosis (SSc), systemic lupus erythematosus (SLE), dermatomyositis (DM) or scleroderma spectrum disorder (SSD), and in healthy control subjects (HC) measured by real-time PCR. miR-142-3p concentrations are shown on the ordinate. The horizontal dotted line indicates the cut-off levels, and bars show means. dcSSc, Diffuse cutaneous SSc; lcSSc, limited cutaneous SSc. * $P < 0.001$ compared with the values in HC, SLE, DM and SSD.

with elevated miR-142-3p levels tended to have a short sublingual frenulum. We could not find significant differences in any other clinical or laboratory features between patients with and without elevated miR-142-3p levels. Considering that a short sublingual frenulum is thought to be caused by fibrosis of the lingual frenulum, serum miR-142-3p levels may be correlated with the activity of tissue fibrosis in this disease.

Discussion

We expected that serum miRNA might be a marker in collagen disease, and that serum miR-142-3p levels would be downregulated in SSc sera because αV integrin, the target of the miRNA, is upregulated in SSc. However, contrary to our expectation, we found that serum miR-142-3p levels were significantly higher in patients with SSc than in healthy control subjects or in patients with SSD, SLE or DM. The increased serum miR-142-3p in patients with SSc may be due to negative feedback against the increased integrin αV expression in dermal fibroblasts. Alternatively, the increased miR-142-3p may contribute to the pathogenesis of this disease by downregulating other targets. This finding needs to be clarified in future studies.

To our knowledge, this study is the first to examine serum miRNA levels using both SSD and SSc sera. The concept of SSD was originally proposed by Maricq *et al.*¹⁸ to unify the conditions of typical SSc, early forms

Table 1 Correlation of serum miR-142-3p levels with clinical and serological features in patients with systemic sclerosis (SSc).

	Patients with		<i>P</i>
	Normal 142-3p levels (<i>n</i> = 29)	Elevated 142-3p levels (<i>n</i> = 32)	
Mean age at serum sampling, years	60.8	67.4	0.29
Mean duration of disease, years	6.3	6.6	0.96
Type, diffuse/limited	12/17	11/21	0.38
m-TSS, score	11.0	11.5	0.86
Clinical features			
Pitting scars/ulcers	41.4	40.6	0.63
Nailfold bleeding	44.8	40.6	0.47
Raynaud phenomenon	79.3	78.1	0.58
Telangiectasia	24.1	21.9	0.54
Contracture of phalanges	72.4	65.6	0.39
Calcinosis	0	0	
Diffuse pigmentation	27.6	15.6	0.20
Short SF	62.1	40.6	0.08
Sicca symptoms	37.9	28.1	0.29
Organ involvement			
Pulmonary fibrosis	34.5	37.5	0.51
Mean %VC	97.7	99.2	0.79
Mean % DL _{CO}	73.1	75.5	0.66
Pulmonary hypertension	13.8	15.6	0.57
Oesophagus	20.7	18.8	0.55
Heart	41.4	28.1	0.21
Kidney	0	0	–
Joints	24.1	31.2	0.37
Thrombosis	0	0	–
ANA specificity			
Topo I	17.2	25.0	0.34
ACA	34.5	43.8	0.32
U1 RNP	13.8	0.1	0.44

ACA, anticentromere antibody; ANA, antinuclear antibody; DL_{CO}, diffusion capacity for carbon monoxide; m-TSS, modified Rodnan Total Skin Thickness Score; RNP, ribonucleoprotein; SF, sublingual frenulum; Topo I, anti-topoisomerase I antibody; VC, vital capacity. Unless indicated, values are percentages. *P*-values were calculated using the Fisher exact probability test or Student *t*-test.

of SSc, and closely related disorders including mixed connective-tissue disease. Later, Ihn *et al.*¹⁹ defined patients with SSD as those who did not fulfil the criteria of SSc but might develop SSc in the future, and established a new diagnostic method using the points system described above to distinguish patients with SSD from those with early SSc. It can be difficult to distinguish early SSc from SSD, because skin sclerosis is sometimes not apparent in early SSc. Progressive fibrosis of SSc is often irreversible, at least clinically,

thus, there is an urgent need to develop new strategies to diagnose patients as early as possible and allow careful monitoring. To allow this, the concept of SSD needs to be further understood and characterized. In this study, we found a significant difference in miRNA levels between patients with SSD and patients with SSc, hence, serum levels of miR-142-3p levels may be useful as the diagnostic marker for the differentiation of SSc from SSD. Moreover, we frequently encounter patients with SSD who are at with increased risk of future development of SSc. Serial time-course measurement of miR-142-3p concentration in patients with SSD might lead to early detection of developing SSc.

Although specific elevation of miR-142-3p levels in patients with SSc indicate that this miRNA plays an important role in the pathogenesis of fibrosis seen in SSc, and that serum miR-142-3p levels are correlated with disease activity, we could not find a significant correlation between serum miR-142-3p levels and the clinical features of SSc. This may be because of the small number of patients, thus larger studies are needed.

Conclusion

Our data indicate that serum levels of miR-142-3p may be elevated specifically in patients with SSc, correlating with the severity of this disease, and may be useful diagnostic markers for presence of SSc and for the differentiation of SSc from SSD.

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