microRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma

Takaomi Sing1, Masatoshi Jinnin1, Keitaro Yamane1, Norihito Honda1, Kastunari Makino1, Ikko Kajihara1, Takamitsu Makino1, Keisuke Sakai1, Shinichi Masuguchi1, Satoshi Fukushima1 and Hironobu Ihn1

Abstract

Objectives. microRNAs (miRNAs) play a part in various cellular activities. However, the role of miRNA in SSc is not fully understood. This study investigated the expression and role of miR-92a in SSc patients and evaluated the possibility that miR-92a is involved in the pathogenesis of this disease.

Methods. Serum samples were obtained from 61 SSc patients. mRNAs were purified from serum and levels of miR-92a and miR-135 were measured with quantitative real-time PCR. miR-92a expression in dermal fibroblasts was also determined by quantitative real-time PCR. Immunoblotting was performed to detect MMP-1 protein.

Results. The median serum levels of miR-92a, not miR-135, were significantly higher in SSc patients than normal subjects. The constitutive up-regulated miR-92a expression was also found in cultured dermal fibroblasts from SSc skin, which was decreased by the transfection with siRNA of TGF-β1. Furthermore, the forced overexpression of miR-92a in normal dermal fibroblasts using miR-92a mimic resulted in the down-regulation of MMP-1 expression.

Conclusion. The increase of miR-92a in SSc may be due to the stimulation of intrinsic TGF-β1 activation seen in this disease. There is also a possibility that MMP-1 is the target of miR-92a and that increased miR-92a expression therefore plays a role in excessive collagen accumulation in SSc via the down-regulation of MMP-1 expression.

Key words: collagen disease, PCR, integrin.

Introduction

SSc is an autoimmune disorder characterized by cutaneous and visceral fibrosis. Such fibrosis may result from the activation of fibroblasts triggered by vascular dysfunction, immune dysregulation and inflammation, but its mechanism is still unknown [1, 2]. However, many characteristics of the activated fibroblasts from SSc skin resemble those of healthy fibroblasts stimulated by exogenous TGF-β1, one of the most potent stimulatory cytokines of extracellular matrix production [3, 4]. SSc fibroblasts produce excessive amounts of various collagens [4, 5], while the blockade of TGF-β1 signalling normalizes the increased collagen expression in these cells [6], suggesting that the activation of dermal fibroblasts in SSc may result from the intrinsic activation of TGF-β1 signalling.

We previously reported that αvβ5 and αvβ3 integrins are overexpressed in SSc fibroblasts, which cause the activation of TGF-β1 signalling [7–10]. Integrin overexpression is thought to be the most upstream event in the process of the self-activation of SSc fibroblasts. However, the factors that mediate integrin overexpression in SSc have yet to be clarified.

We focused on microRNA (miRNA) in investigating integrin overexpression. miRNAs, short ribonucleic acid molecules an average of only 22 nucleotides long, are post-transcriptional regulators that bind to complementary sequences in the three prime untranslated regions (3′-UTRs) of mRNAs, leading to gene silencing. Among
them, miR-92a can bind to the 3′-UTR of the integrin αv subunit based on miRNA target gene predictions using TargetScan (version 5.1; http://www.targetscan.org/), a bioinformatics program [11]. Many studies have shown serum miRNA levels to be a useful biomarker for diagnosis, prognosis and therapy, especially in various malignant tumors. Therefore this study investigated the serum levels of miR-92a in SSc patients and tried to evaluate the possibility that miR-92a is involved in the pathogenesis of this disease.

Materials and methods

Clinical assessment and patients’ material

Serum samples were collected from 61 patients with SSc (12 males and 49 females; age range 29–85 years). All patients were grouped according to the classification system designed by LeRoy et al. [12]; twenty-three patients had dcSSc and 38 patients had lcSSc, as previously described [13]. All patients fulfilled the criteria proposed by the ACR [14]. Clinical and laboratory data shown in this study were recorded at the time of serum sampling. The patients were assessed for the presence of gastrointestinal, pulmonary, cardiac or renal involvement, as previously described [13]. Control serum samples were obtained from 18 healthy age- and sex-matched volunteers. Seven patients with DM, 7 patients with SLE and 12 patients with scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc, but who were considered to be at risk for developing SSc in the future based on the criteria described below, were also included in this study [15–17]. The study was approved by the Ethics Review Committee at Kumamoto University (No. 177). Written informed consent was obtained before the patients and healthy volunteers were entered into this study according to the Declaration of Helsinki. All serum samples were stored at −80°C prior to use.

miRNA extraction

miRNA isolation from cultured cells was performed using the RT2 qPCR-Grade miRNA Isolation Kit (SA Bioscience, Frederick, MD, USA). miRNA was isolated from serum samples using an miRNeasy RNA Isolation Kit (Qiagen, Valencia, CA, USA), following the manufacturer’s instructions with minor modifications [18–22]. Briefly, 100 µl of serum was supplemented with 5 µl of 500 µmol/µl synthetic non-human miRNA (Caenorhabditis elegans miR-39; Takara Bio Inc., Shiga, Japan) as controls, providing an internal reference for normalization of technical variations between samples. After Qiazol solution (1 ml) was added and mixed by vortexing, samples were incubated at room temperature for 5 min. Aqueous and organic phase separation was achieved through the addition of chloroform. The aqueous phase was applied to an RNeasy spin column and an RNeasy MinElute spin column. miRNA was eluted from the column with nuclease-free water.

Quantitative real-time PCR

miRNAs from cultured cells were reverse transcribed into first strand cDNA using an RT² miRNA First Strand Kit (SA Biosciences). For quantitative real-time PCR, primers for miR-92a or U6 (SA Biosciences) and templates were mixed with the SYBR Premix Ex Taq™ (Takara Bio, Inc.). DNA was amplified for 40 cycles of denaturation for 5 sec at 95°C and annealing for 30 sec at 60°C. Data generated from each PCR were analyzed using a Thermal Cycler Dice Real Time System v2.10B (Takara Bio, Inc.). The specificity of the reactions was determined using a melting curve analysis. Transcript levels were normalized to U6.

For cDNA synthesis from serum miRNA, we used mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio, Inc.) [22]. Primers and templates mixed with the SYBR Premix were used for quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800). The primer sequence of hsa-miR-92a, hsa-miR-135 and cel-miR-39 was designed based on miRBase (http://www.mirbase.org). The primer sets were pre-validated to generate single amplicons. DNA amplification was 40 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. The relative fold changes of hsa-miR-92a, hsa-miR-135 and cel-miR-39 were calculated using the standard curve method.

ANAs

ANAs were detected by IIF using HEp-2 cells as the substrate, as previously described [17]. Antibody for topo I, ACA or anti-U1 RNP was determined using a MESACUP TEST Kit for each antibody (MBL, Nagano, Japan) [23].

Diagnostic method of SSD using the point system

For the diagnosis of SSD, a total score was obtained as the sum of the following five factors [15–17]:

(i) Extent of skin sclerosis: 10 points for truncal sclerosis, 5 points for skin sclerosis limited to the extremities and face, 3 points for sclerodactyly alone and 1 point for swollen fingers.

(ii) Pulmonary changes: 4 points for pulmonary fibrosis accompanied by decreased vital capacity (<80%) and 2 points for pulmonary fibrosis accompanied by normal vital capacity (>80%).

(iii) ANA: 5 points for positive anti-Topo I, 3 points for positive ACA or anti-U1 RNP antibody, 2 points for anti-nucleolar antibody and 1 point for other positive ANA.

(iv) Pattern of RP: 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.

(v) Nailfold bleeding (NFB): 2 points for NFB in three or more fingers, and 1 point for NFB in one or two fingers.

A score of 9 or more points is consistent with SSc and a score of 5–8 points is consistent with SSD.

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Cell culture
Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of patients with dcSSc and <2 years of skin thickening, as previously described [7-10]. Control fibroblasts were obtained by skin biopsies from healthy donors. Control donors were matched with SSc patients for age, sex and biopsy site. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 25-cm² culture flasks in modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA). Monolayer cultures independently isolated from different individuals were maintained at 37°C in 5% CO₂ in air. Fibroblasts between the third and six subpassages were used for the experiments. The cells were serum starved for 12–24 h before experiments.

Transient transfection
TGF-β siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). miR-92a mimic was obtained from Qiangen. Control siRNA or control miRNA mimic was also obtained. Lipofectamine RNAiMAX (Invitrogen) was used as the transfection reagent. For reverse transfection, siRNAs or miRNA mimics mixed with transfection reagent were added when cells were plated, followed by incubation for 48 h (for real-time PCR) or 72 h (for Immunoblotting) at 37°C in 5% CO₂. Control experiments showed transcript levels for targets of siRNA to be reduced by >80%, and the expression of miRNA to be increased by at least 5-fold by the transfection of mimic (data not shown).

Cell lysis and immunoblotting
Fibroblasts were washed with cold PBS twice and lysed in Denaturing Cell Extraction Buffer (BIOSOURCE, Camarillo, CA, USA). Aliquots of cell lysates (normalized for protein concentrations) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride filters. The filters were blocked with antibody against MMP-1 (Chemicon, Temecula, CA, USA) or β-actin (Sigma, St Louis, MO, USA). The filters were incubated with secondary antibody, and the immunoreactive bands were visualized using an ECL system (Amersham Biosciences, Arlington Heights, IL, USA) according to the manufacturer’s recommendations.

Statistical analysis
The statistical analysis was carried out with Mann–Whitney U test for the comparison of medians and Fisher’s exact probability test for the analysis of frequency. Correlations were assessed by Pearson’s correlation coefficient. The Bonferroni correction was used for multiple comparisons: P < 0.0071 was considered to be significant for the comparison of serum miR-92a levels. P < 0.0020 or 0.0062 was considered to be significant for the correlation analysis of serum miR-92a levels with the clinical features of patients with SSc or SSD, respectively.

Results

hsa-miR-92a is present in serum
No previous study has demonstrated the expression of hsa-92a in cell-free body fluid. To confirm that the miRNA is actually detectable in human serum, total miRNA was isolated from the serum of healthy individuals and the presence of miR-92a was determined by quantitative real-time PCR using a primer set specific for miR-92a (Fig. 1). hsa-miR-92a was amplified, and C₁ values were increased by the serial dilution of the miRNA. Therefore hsa-miR-92a is likely to be present in the serum and quantitative using our method.

Serum levels of hsa-miR-92a
Fig. 2 depicts the serum miR-92a levels in 61 SSc patients. Seven DM patients, 7 SLE patients and 12 SSD patients who did not fulfill the criteria of SSc but were thought likely to develop SSc in the future were also included in the study [15-17].

The median serum miR-92a levels were significantly higher in SSc patients than in normal control subjects (P < 0.00001 by Mann–Whitney U test). Classification of SSc patients into lcSSc and dcSSc as described in the Materials and methods section revealed that the median serum levels in both lcSSc and dcSSc patients were significantly increased in comparison with those in normal subjects (P < 0.00001), whereas we could not find a significant difference in the values between dcSSc and lcSSc patients.

On the other hand, the median miR-92a levels in the 7 DM patients, 7 SLE patients and 12 SSD patients were slightly elevated in comparison with those in normal subjects, but not significantly. miR-92a levels in SSD patients were significantly lower than those in SSc (P < 0.003). Taken together, the serum miR-92a levels tended to be increased only in SSc patients. When the cut-off value was set at the mean (2 s.d.) of the normal subjects, increased serum levels of miR-92a were found in 29 of the 61 (47.5%) SSc patients and 3 of the 12 (25.0%) SSD patients.

We also determined the serum levels of miR-135, another putative regulator of integrin αv based on TargetScan. As shown in Fig. 3, miR-135 was also found in the serum, but was detected in only 3 of 18 normal subjects (16.7%), 3 of 12 SSD patients (25.0%) and 5 of 61 SSc patients (8.2%). The median serum miR-135 levels were not different among SSc patients, SSD patients and normal subjects, and there was no correlation between serum miR-92a levels and miR-135 levels by Pearson’s correlation coefficient in both normal subjects and patients with SSc or SSD. Therefore miR-92a seems to be specifically increased in the sera of SSc patients.
Correlation of serum miR-92a levels with clinical and laboratory features in patients with SSc or SSD

Table 1 represents the association of serum miR-92a levels with the clinical features of patients with SSc or SSD. We found that SSc patients with increased miR-92a levels (above the cut-off value) tended to have telangiectasia at a lower frequency than those with normal levels (10.3% vs 31.3%, \( P < 0.05 \) by Fisher's exact probability test), but the difference was insignificant when \( P < 0.0020 \) was considered to be significant according to the Bonferroni correction. There was no statistically significant difference in terms of other clinical or laboratory features, including skin score or pulmonary fibrosis between SSc patients with and without increased miR-92a levels.

In SSD patients, the points calculated by the diagnostic method of SSD were not correlated with the levels of miR-92a (\( r = -0.39, P = 0.19 \)) by Pearson's correlation coefficient. We could not find a significant difference between SSD patients with and without increased

**Fig. 1** hsa-miR-92a is detected in serum.

Serial dilution of cDNA (as is, 10-fold dilution, 100-fold dilution and 0) synthesized from serum miRNA was amplified by PCR with the hsa-miR-92a primer. Amplification curves of gene-specific transcripts are shown to figure the exponential increase of fluorescence. The horizontal dotted line indicates the threshold.

**Fig. 2** Expression of miR-92a in the sera of patients with DM, SLE, SSD or SSc, and of normal subjects.

miR-92a levels measured as described in the Materials and methods section are shown on the ordinate. The maximal value in SSc patients was set at 1. The horizontal dotted line indicates the cut-off level, Bars show medians. \( P \)-values were determined using a Mann-Whitney U test. \( ^*P<0.05 \) as compared with the value in normal subjects.

**Fig. 3** Serum levels of miR-135 in patients with SSD or SSc, and in normal subjects.

The maximal value in SSc patients was set at 1, as described in Fig. 2.
miR-92a levels in the frequency of clinical features such as NFB or RP. Therefore the serum miR-92a levels are not likely to be a specific marker for clinical manifestations of SSD.

Functional studies of miR-92a

Lastly, we tried to clarify upstream pathways of the increased miR-92a in SSc. miR-92a expression was significantly increased in normal cultured dermal fibroblasts in the presence of TGF-β and SSc fibroblasts in comparison with untreated normal fibroblasts \( (P < 0.05, \text{Fig. 4a}) \). Inhibition of TGF-β signalling by specific siRNA in SSc fibroblasts down-regulated miR-92a expression \( (P < 0.05; \text{Fig. 4a}) \). Constitutively, up-regulated miR-92a expression in SSc fibroblasts is consistent with increased serum miR-92a in SSc patients, indicating the possibility that dermal fibroblasts are one of the cellular sources of serum miR-92a. The normalization of miR-92a levels by TGF-β siRNA in SSc fibroblasts suggests that an increase of miR-92a is due to stimulation by intrinsic TGF-β activation seen in this cell type, as described in the Introduction section.

We also evaluated the consequences of up-regulated miR-92a in SSc fibroblasts. Forced overexpression of miR-92a in normal fibroblasts using miR-92a mimic resulted in the down-regulation of MMP-1 expression (Fig. 4b), suggesting that MMP-1 is the target of miR-92a and that increased miR-92a expression plays a role in the excessive collagen accumulation in SSc fibroblasts via the down-regulation of MMP-1.

Discussion

Over 1000 miRNAs are thought to be present in the human genome, and they may target ~60% of mammalian genes [24]. It has been revealed by recent research that miRNAs

<table>
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<tr>
<th>TABLE 1 Correlation of serum miR-92a levels with clinical and serological features in patients with SSc or SSD</th>
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<tr>
<td><strong>Serum miR-92</strong></td>
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<tr>
<td><strong>SSc patients with increased miR-92a levels (n = 29)</strong></td>
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<td><strong>SSc patients with normal miR-92a levels (n = 32)</strong></td>
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<td><strong>SSD patients with increased miR-92a levels (n = 3)</strong></td>
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<td><strong>SSD patients with normal miR-92a levels (n = 9)</strong></td>
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<td>U1 RNP</td>
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Unless indicated, values are percentages. The points were calculated by the diagnostic method of SSD as described in the Materials and methods section. MRSS: modified Rodnan total skin thickness score; SF: sublingual frenulum; VC: vital capacity; DLco: diffusion capacity for carbon monoxide; U1 RNP: anti-U1 RNP antibody. *\( P < 0.05 \) vs patients with normal 92a levels using Fisher’s exact probability test.
miR-92a in SSc

Fig. 4 miR-92a expression is regulated by TGF-β signaling in SSc fibroblasts.

(a) Levels of miR-92a in normal fibroblasts in the presence or absence of TGF-β and in SSc fibroblasts with or without TGF-β siRNA (n = 3). The value in normal fibroblasts transfected with control siRNA without TGF-β stimulation was set at 1. *P < 0.05. (b) Normal human fibroblasts at a density of 2 x 10^4 cells/well in 24-well culture plates were transfected with control miRNA mimic or miR-92a mimic for 72 h. Cell lysates were subjected to immunoblotting using MMP-1 antibody.

We expected that serum miRNA could also be useful as a marker in collagen disease and that serum miR-92a levels are decreased in SSc patients because miR-92a is a putative down-regulator of integrin, which is up-regulated in SSc. Contrary to expectations, however, median serum miR-92a levels were observed to be significantly higher in SSc patients than in normal subjects. We theorize that such an increase of miR-92a in SSc may be caused by intrinsic activation of TGF-β, and may be induced as a negative feedback mechanism against the overexpressed integrin. Another possibility is that increased miR-92a may contribute to the pathogenesis of this disease by regulating other targets. The overexpression of miR-92a results in the down-regulation of MMP-1. MMP-1 down-regulation in SSc has been reported and it may further the excess accumulation of collagen in this disease by decreasing collagen degradation [27], indicating that the overexpression of miR-92a plays a role in the pathogenesis of SSc through the down-regulation of MMP-1. Since MMP-1 is not predicted to be a target of miR-92a by the bioinformatics program described above, miR-92a may regulate MMP-1 expression indirectly via other targets.

We found that high levels of miR-92a are associated with SSc, whereas moderately raised levels are associated with connective tissue diseases in general. Serum miR-135 was not up-regulated in SSc patients. Therefore serum miR-92a levels may be specifically increased in SSc patients in comparison with normal subjects but a sensitivity of 47.5% is unlikely to be clinically useful. Serum miR-92a levels may have the potential to be used alongside other parameters in diagnostic algorithms.

In this study the serum samples of SSc patients were taken from those who easily fulfill the clinical criteria for this disease. Before the test can be used in a clinical setting, it needs to be examined longitudinally in a cohort of patients in whom the disease is in its early stages. The concept of SSD was originally proposed by Maricq et al. [15, 16] to unify typical SSc, early forms of SSc and closely related disorders, including MCTD. Following this, Ihn et al. [17] redefined SSD as occurring in patients who did not fulfill the criteria of SSc but were thought likely to develop SSc in the future, and proposed a new method using a points system to diagnose SSD patients earlier and follow-up carefully against the development of SSc. In this study we found a significant difference in the miR-92a levels between SSD and SSc patients. Serum levels of miR-92a levels may be useful for the differentiation of SSc from SSD, and serial time-course measurement of miR-92a levels in SSD patients may lead to early detection of developing SSc.

We also found that the prevalence of telangiectasia in SSc patients with increased miR-92a levels tended to be lower than in those with normal levels. Vascular damage is one of the earliest events in SSc and, as described above, this damage may contribute to the fibroblast activation and tissue fibrosis seen in this disease. Although the mechanisms of the decreased frequency of telangiectasia by increased miR-92a are unknown, there is a possibility that MMP-1 is involved in the formation of telangiectasia and increased miR-92a may regulate telangiectasia formation negatively via the down-regulation of MMP-1. Clarifying the role of miRNAs in SSc may help to achieve a better understanding of this disease and lead to the development of new therapeutic approaches.

**Rheumatology key messages**

- Median serum miR-92a levels were higher in scleroderma patients than in normal subjects.
- miR-92a was constitutively overexpressed in cultured dermal fibroblasts from SSc skin.
- The forced overexpression of miR-92a mimics in normal dermal fibroblasts down-regulated MMP-1 expression.
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