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Serum microRNA screening and functional studies reveal miR-483-5p as a potential driver of fibrosis in systemic sclerosis

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ABSTRACT

Objective: MicroRNAs (miRNAs) are regulatory molecules, which have been addressed as potential biomarkers and therapeutic targets in rheumatic diseases. Here, we investigated the miRNA signature in the serum of systemic sclerosis (SSc) patients and we further assessed their expression in early stages of the disease.

Methods: The levels of 758 miRNAs were evaluated in the serum of 26 SSc patients as compared to 9 healthy controls by using an Openarray platform. Three miRNAs were examined in an additional cohort of 107 SSc patients and 24 healthy donors by single qPCR. MiR-483-5p expression was further analysed in the serum of patients with localized scleroderma (LoS) (n = 22), systemic lupus erythematosus (SLE) (n = 33) and primary Sjögren's syndrome (pSS) (n = 23). The function of miR-483-5p was examined by transfecting miR-483-5p into primary human dermal fibroblasts and pulmonary endothelial cells.

Results: 30 miRNAs were significantly increased in patients with SSc. Of these, miR-483-5p showed reproducibly higher levels in an independent SSc cohort and was also elevated in patients with preclinical-SSc symptoms (early SSc). Notably, miR-483-5p was not differentially expressed in patients with SLE or pSS, whereas it was up-regulated in LoS, indicating that this miRNA could be involved in the development of skin fibrosis. Consistently, miR-483-5p overexpression in fibroblasts and endothelial cells modulated the expression of fibrosis-related genes.

Conclusions: Our findings showed that miR-483-5p is up-regulated in the serum of SSc patients, from the early stages of the disease onwards, and indicated its potential function as a fine regulator of fibrosis in SSc.

1. Introduction

Systemic sclerosis (SSc) is a rare, systemic autoimmune disorder characterized by vascular damage, immune activation and fibrosis of the skin and/or internal organs [1]. Even if the pathogenesis of

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SSc still remains elusive, increasing evidence suggests that microvascular injury and endothelial cell activation are the earliest events in the evolution of the disease [2]. The complex interplay between altered endothelial cells and immune cells infiltrating the tissues results in the secretion of inflammatory cytokines and profibrotic mediators, leading to myofibroblast transition and culminating in excessive deposition of extracellular matrix (ECM), defined as fibrosis, the hallmark of SSc [3–5]. Activated myofibroblasts in fibrotic tissue can derive from different precursors including activated fibroblasts, pericytes, fibrocytes, epithelial cells and endothelial cells, and are characterized by *de novo* expression of α-smooth muscle actin (αSMA) [6].

Recently, microRNAs (miRNAs) were proposed as possible novel players in SSc fibrosis, being capable of modulating several fibrotic-related genes [7,8]. MiRNAs constitute a class of short non-coding RNAs (19–24 nucleotides) that regulate gene expression at the post-transcriptional level. Beside their intracellular function, miRNAs are also present in biological fluids, where they circulate bound to protein complexes or enclosed in extracellular vesicles, including exosomes [9]. It has been demonstrated that miRNAs embedded in exosomes are involved in cell-to-cell communication by transferring their content into the recipient cells [9,10]. Furthermore, circulating miRNAs are often indicated as potential biomarkers and therapeutic targets in several diseases, since they are highly stable molecules and their aberrant expression frequently reflects the pathophysiology of diseases [11,12]. MiRNAs have been shown to be altered in the serum of SSc patients, implicating them as possible diagnostic biomarkers for SSc and potential players in the disease pathogenesis [7,13,14].

Hitherto, the knowledge on circulating miRNAs in SSc is limited and a broad profiling of circulating miRNAs is lacking. Moreover, the expression of secretory miRNAs in early SSc patients, who do not yet present with skin fibrosis, has not been explored thus far. Identifying molecular changes at the early stages is of utmost importance, as an understanding of the pathophysiological pathways leading up to disease could open novel avenues for therapeutic intervention before the actual onset of fibrosis.

Here, we present a comprehensive profiling of 758 miRNAs in the serum of SSc patients. MiR-483-5p was demonstrated to be consistently upregulated in two independent SSc cohorts and showed high expression before the onset of fibrosis. Moreover, functional experiments indicated the potential implication of miR-483-5p in the modulation of fibrosis.

2. Material & methods

2.1. Patients

Blood from patients and sex- and age-matched healthy controls (HC) was obtained from the University Medical Center Utrecht in the Netherlands and the Scleroderma Unit of the Fondazione IRCCS Policlinico of Milan in Italy. All patients signed a consent form approved by the local institutional review boards, prior to participation in the study. Samples and clinical information were treated anonymously immediately after collection. Patients fulfilling the ACR/EULAR 2013 classification criteria [15] were classified in relation to the extent of skin fibrosis as limited cutaneous (lcSSc) or diffuse cutaneous SSc (dcSSc) [16]; patients fulfilling the classification criteria without skin fibrosis will be referred to as non-cutaneous SSc (ncSSc) throughout the manuscript. Additionally, early SSc (eaSSc) subjects were defined as patients presenting with Raynaud's phenomenon (RP) and SSc-specific autoantibodies and/or typical nailfold videocapillaroscopy (NVC) abnormalities [17]. The presence of interstitial lung disease (ILD) was identified as typical involvement of the lung parenchyma >5% on high resolution CT [18] accompanied by a reduced forced vital capacity (FVC) or a diffusing capacity for carbon monoxide (DLco) < 80% of predicted values [19].

Two separate cohorts were recruited for the current study (Discovery and Validation Cohort) at the University Medical Center Utrecht and the Scleroderma Unit of Fondazione IRCCS Policlinico of Milan. Early SSc patients were also included in the validation cohort. Demographics and clinical characteristics of the patients are depicted in Table 1.

2.2. Blood collection and RNA extraction

Blood was collected in a Vacutainer® SST II tube (BD Vacutainer) and serum was isolated after centrifugation at 1500*g* for 10 min at room temperature and stored at −80 °C prior to use. Serum RNA was extracted from 200 μl of serum using the miRcury RNA Isolation kit for Biofluids (Exiqon), according to the manufacturer's instructions. During extraction, 300 pg of a non-human synthetic miRNA (Arabidopsis thaliana ath-miR-159a) was added to each sample as a spike-in to monitor the technical variability during the isolation and for subsequent data normalization.

RNA from cells was isolated using the Allprep Universal Kit (Qiagen), according to the manufacturer's instructions. The concentration of RNA was determined using a Qubit 2.0 fluorimeter (Invitrogen) and the Qubit RNA HS Assay Kit (Molecular Probes, Life Technologies).

2.3. MiRNA profiling

The screening of 758 miRNAs in the Discovery cohort was performed by TaqMan RT-qPCR on an OpenArray platform (LifeTechnologies) according to manufacturer's instructions with minor adjustments. 2.5 μl of isolated serum RNA was reverse-transcribed by using the miRNA megaplex RT primer pools (LifeTechnologies). cDNA was pre-amplified using the Megaplex PreAmp Primer pools in the presence of the TaqMan PreAmp Master mix (Life Technologies), by using the following thermal cycler conditions: 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min and 16 cycles of 95 °C for 15 s and 60 °C for 4 min and one single cycle of 96 °C for 10 min. The miRNA OpenArray profiling was performed on the amplified cDNA diluted 1:40 in 0.1x TE (pH8.0), by using the Taqman OpenArray master mix on the QuantStudio 12k flex Real-Time PCR system (LifeTechnologies). The Relative Quantification method of the GeneExpression Suite Software (ThermoFisher) was applied to analyze the data by using the comparative threshold cycle method (Crt). Low expressed miRNAs were excluded from the analysis: Crt was set at 27 maximum and only miRNAs with high amplification quality (>1.24) were taken into consideration. The expression of each miRNA was calculated after normalization on the levels of the exogenous spike-in ath-miR-159a and expressed as Fold Change (FC) as compared to the control samples. MiRNA profiling data have been deposited in NCBI's Gene Expression Omnibus (GSE) and are accessible through GEO Series accession number GSE108918.

2.4. MiRNA and gene expression analysis

The validation of miRNA expression in serum was performed by single Taqman miRNA RealTime-quantitative PCR (RT-qPCR) assays (LifeTechnologies). Briefly, 2.5 μl of serum RNA was reverse-transcribed using the specific microRNA assays (ath-miR159a; ID 000338, has-miR-501-3p; ID 002435, has-miR-483-5p; ID 002338, U6 snRNA; ID 001973, Life Technologies) and measured with the specific TaqMan assay on the QuantStudio 12k flex System, in the presence of the TaqMan Fast Advanced Master mix (LifeTechnologies), following manufacturer's instructions. MiRNA

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