Comparison of serum exosome miRNA from patients with Raynaud's phenomenon with positive and negative serum antinuclear antibodies

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Abstract

Objective

To compare the microRNAs (miRNAs) contained within serum exosomes isolated from patients with Raynaud's phenomenon (RP) and negative antinuclear antibodies (ANA) to the miRNA contained in serum exosomes isolated from patients with RP and positive ANA.

Methods

Serum exosomes were isolated employing a polymer precipitation procedure. Next Generation Sequencing (NGS) was used to identify the miRNAs contained in the exosomes isolated from the two clinical cohorts and to analyse the differences in their contents.

Results

The NGS results identified six miRNAs that displayed significant differences in their content between serum exosomes from patients with RP with negative serum ANA compared to miRNAs contained in serum exosomes from patients with ANA-positive RP.

Conclusion

A comparative analysis of miRNAs contained within serum exosomes of patients with RP and negative ANA vs. samples from patients with RP and positive ANA identified several differentially expressed miRNAs that may represent non-invasive biomarkers to assist in the identification of patients with RP at risk of evolving into systemic sclerosis.

Key words

Raynaud's phenomenon, ANA, microRNA, serum exosomes

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Introduction

Systemic sclerosis (SSc) is an idiopathic autoimmune disease characterised by multiple immunological, vascular, and fibroproliferative abnormalities that result in a wide and heterogeneous spectrum of clinical manifestations (1-3). The disease has an overall poor prognosis causing serious disability, high morbidity, and increased mortality (1-3). Vasculopathy and systemic fibroproliferative alterations are hallmark pathological processes in SSc that are responsible for the severity of the disease and determine its clinical outcome and mortality (3-5). The vascular alterations are among the earliest SSc clinical manifestations and initially present as Raynaud's phenomenon (RP) often preceding any evidence of a fibrotic or autoimmune process (6-8).

RP is a relatively common clinical manifestation with a prevalence in the general population of approximately 5%. Primary RP occurs as an isolated clinical entity not associated with any autoimmune or inflammatory disease (6-8). However, it is well recognised that a minor proportion of patients with RP may eventually evolve into SSc. Besides a positive serum autoantibody test, the patients with RP who evolve into SSc initially display minimal clinical evidence of cutaneous or visceral fibrotic changes. Indeed, based on these observations, it has been recently suggested that these patients may represent a novel clinical subset of SSc that has been termed "Very Early SSc" (9-14). The importance of recognising the patients at risk of evolving into SSc, referred here as to pre-SSc patients, must be strongly emphasised owing to the fact that establishing the diagnosis of these pre-SSc patients will assure that they are treated and managed appropriately prior to the appearance of clinical alterations of the disease resulting in a decrease in SSc-related symptoms and overall SSc mortality.

Extensive recent investigations have shown that several molecular pathways of SSc pathogenesis involve microRNAs and have indicated that microRNAs play an important role in the development and progression of the disease (15-22). Indeed, it has been suggested that further characterisation of the functional and molecular effects of miRNAs in SSc will very likely provide valuable information for SSc biomarker discovery, as well as, for the identification of novel and effective therapeutic approaches for the disease (21, 22). The mechanisms involved in gene expression regulation by miRNAs are highly complex and require the sequence-specific complementary binding of the miRNA to the 3′ or 5′ untranslated region (UTR) of target mRNAs leading to either inhibition of mRNA translation or increased mRNA degradation (15).

Exosomes are secreted microvesicles released essentially by all living cells into the extracellular space that eventually may enter the blood stream. Numerous studies have shown that these microvesicles play crucial roles in cellular physiology and are important mediators of numerous pathological processes including SSc and other fibrotic diseases (23-26). Exosomes contain a large array of macromolecules including mRNAs, miRNAs, proteins, cytokines, chemokines and growth factors. The macromolecular content of exosomes is determined by their cell of origin and, most importantly, reflects the functional or pathologic status of the originating cells. Accordingly, extensive studies have shown that analysis of the macromolecular content of exosomes provided novel insights into the pathogenesis of numerous diseases and allowed the identification of disease-specific biomarkers (23-26). Furthermore, numerous recent studies have shown that exosomes are involved in the development of tissue fibrosis and may play a role in SSc pathogenesis (27, 28).

Materials and methods

Serum samples from patients included in the study

Serum samples were retrieved from the Thomas Jefferson Scleroderma Center Serum Bank that contains de-identified and coded patient serum samples that were obtained following a protocol approved by the Thomas Jefferson University Institutional Review Board (IRB) in accordance with the Declara-

tion of Helsinki. All patients who participated in this study provided written informed consent for their inclusion and all patients fulfilled the accepted diagnostic criteria for RP based on the presence of biphasic or triphasic vasospastic manifestations occurring following cold exposure (6-8). Five patients with RP who had negative results for serum antinuclear antibodies (ANA) in repeated testing and four patients with typical symptoms of RP and positive antinuclear antibodies (ANA) in their serum comprised the two cohorts of the study. The demographic and relevant clinical features of the patients studied are shown in Table I. None of the patients displayed any clinical manifestations of SSc or other autoimmune disease, although regrettably, most of these patients did not undergo nailfold capillary microscopic examination at the time the blood samples were obtained.

Isolation of serum exosomes

Exosomes were isolated from the serum samples utilising ExoQuick (System Biosciences), a polymer-based precipitation agent that specifically precipitates exosomes in a native state and with high purity (29-31). We previously validated that the exosomes isolated using ExoQuick were within the expected exosome 80-150 nm diameter and were free of larger diameter vesicle contaminants (28). The isolated exosomes were also analysed for exosome marker proteins employing Western blots and by transmission electron microscopy (28).

Characterisation of mature RNA transcripts and miRNAs in serum exosomes

Total RNA was isolated from exosomes obtained from serum samples from both patient cohorts and exosome RNA was purified using SeraMir RNA columns following the procedures suggested by the manufacturer, as described previously (28). RNA concentration, purity and integrity were determined utilising the Agilent bio-analyser. In preliminary studies performed to examine the feasibility of the study described in this report, we found that samples obtained from a large number of patients did not yield sufficient miRNA for subsequent analysis, therefore, these samples were not included in the final study.

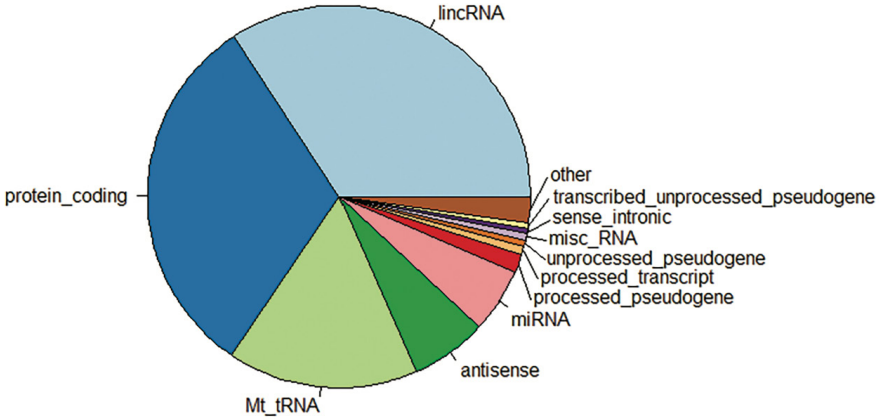

Fig. 1. Serum exosome transcript distribution by Ensembl biotype. The pie chart represents the proportion of transcript types based on transcripts per million (TPM) averaged across all samples.

Table I. Demographic and clinical features of Raynaud's phenomenon patients studied.

Patient number	Diagnosis	Age	Gender	Ethnic group	ANA titre	ANA pattern
1	ANA - RP	21	F	C	NEG	-
2	ANA - RP	40	F	C	NEG	-
3	ANA - RP	29	F	C	NEG	-
4	ANA - RP	24	F	C	NEG	-
5	ANA - RP	30	F	C	NEG	-
6	ANA + RP	47	F	C	>1:160	Homogeneous
7	ANA + RP	51	F	C	1:5120	Speckled/Nucleolar
8	ANA + RP	36	F	C	>1:160	Homogeneous
9	ANA + RP	47	F	C	1:640	Nucleolar

ANA negative Raynaud's phenomenon (ANA – RP) *vs.* ANA positive Raynaud's phenomenon (ANA + RP).

Library sequencing and preprocessing

Small RNA libraries were prepared and analysed at the Cancer Genomics and Bioinformatics Laboratory, Department of Pharmacology, Physiology and Cancer Biology, Sidney Kimmel Cancer Center of Thomas Jefferson University under the supervision of Adam Ertel and Paolo Fortina. The libraries were prepared using the Takara SMARTer smRNA-Seq Kit and sequenced on the Illumina NextSeq 500 instrument using a single-end 75 cycle flowcell. Single-end 75-base sequence reads were pre-processed employing the SMARTer smRNA-Seq Kit for Illumina analysis by trimming extraneous bases added during sequencing library preparation including the first three positions containing random nucleotides and poly-adenylated tail-end sequences as described previously (32). Sequence reads shorter than 15 nucleotides were removed during filtering.

Analysis of miRNA sequencing and differential expression

These analyses were also performed at the Center for Cancer Genomics. Two approaches were used to analyse the exosome-derived transcripts: the first assessed mature miRNA content using the miRDeep2 pipeline, whereas the second approach assessed the whole transcriptome content using the RSEM-STAR pipeline. The mirDeep2 pipeline was used to align the pre-processed reads to the latest miRBase 22 miRNA sequence definitions from miRbase human genome assembly version GRCh38 and estimate both mature miRNA counts, and normalised values expressed as counts per million mapped miRNA reads (RPM) as described previously (32). The RSEM-STAR pipeline was used to align the pre-processed reads to the whole human transcriptome based on the human genome assembly version GRCh38 and GENCODE version 30 human gene feature annotations. Whole transcriptome alignments were used to assess the distribution of

Table II. The 15 topmost miRNAs detected in normal human serum exosomes compared with RPM and rankings reported in the EVmiRNA database.

Mature miRNA	Total read count	average RPM ($x10^3$)	EVmiRNA RPM	Rank in EV miRNA database
hsa-miR-1246	399,846	78.052	315.67	91
hsa-miR-6124	1,540	20.86	NA	NA
hsa-miR-126-5p	7,342	16.37	1977.44	36
hsa-miR-4800-5p	1,026	14.70	NA	NA
hsa-miR-6126	2,617	13.44	2.29	357
hsa-let-7a-5p	6,271	11.72	734.85	62
hsa-let-7b-5p	3,253	6.61	3631.35	22
hsa-miR-486-5p	3,344	66.10	79623.34	1
hsa-miR-423-5p	2,425	6.49	7617.85	12
hsa-let-7f-5p	3,023	6.37	1149.76	49
hsa-miR-483-5p	464	5.45	0.54	441
hsa-miR-191-5p	1,081	4.25	1618.26	40
hsa-miR-142-5p	457	4.06	2529.12	30
hsa-miR-4271	354	3.95	NA	NA
hsa-miR-150-5p	2,123	3.54	438.95	76

Table III. Differences in levels of serum exosome mature miRNA in patients with RP and positive ANA compared with exosome mature miRNA from patients with RP and negative ANA.

Mature miRNa	Base mean ($x10^3$)	log2fold-change	*p*-value
hsa-let-7d-5p	2.292	-3.94	0.02
hsa-miR-106a-5p	1.56	-3.89	0.09
hsa-miR-1273c	2.22	-3.98	0.08
hsa-miR-17-5p	3.39	-5.08	0.01
hsa-miR-4739	2.63	-4.66	0.03
hsa-miR-486-5p	58.97	-2.54	0.04

RNA types contained in the isolated exosomes.

Analysis of the microRNA was performed on mature miRNA normalised RPM values obtained from the miRDeep2 described above. The serum exosome miRNA from patients with RP and positive ANA was compared to the serum exosome miRNA from patients with RP and negative ANA. Owing to the suspected influence of outlier samples, the comparisons were repeated by first removing samples with low Pearson's correlation relative to the other samples, and then, additionally, removing samples with fewer than 5,000 mature miRNA reads. Comparisons were performed between the two groups. Quantitative miRNA analysis was also performed on the whole transcriptome counts generated by the RSEM-STAR pipeline. These comparisons were repeated following removal of suspected outliers.

Results

miRNA analysis

Alignments to both mature miRNA and the human whole transcriptome indicated that various types of RNA molecules

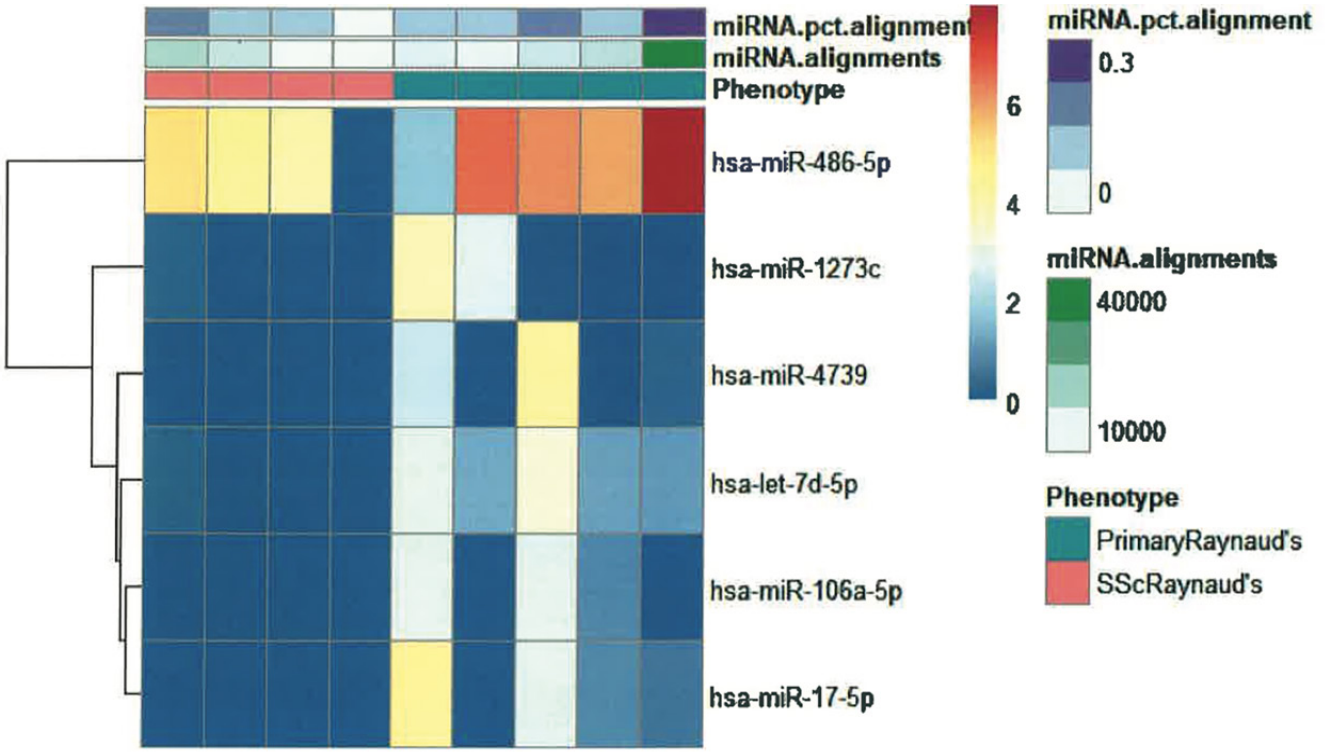

Fig. 2. Heat map of miRNAs displaying differential content in serum exosome samples. The quantitative levels of miRNAs were assessed in exosome samples from patients with RP with positive ANA patients (SSc Raynaud's) and were compared with miRNAs in exosome samples from patients with RP with negative ANA (primary Raynaud's).

Table IV. Differentially expressed RNAs.

A

Gene Symbol	log_2 fold change	p value
NLRP2B	24.11	9.01E-23
CTDP1	8.79	3.99E-05
HEIH	8.06	6.95E-04
MDFI	7.92	8.99E-04
MYOF	7.00	8.18E-04
CHAD	6.99	2.63E-03
MYO15A	6.93	2.85E-03
FAM167B	6.59	4.47E-03
MTRR	6.51	2.26E-03
RPL36AP29	6.45	1.32E-03
FGFRL1	6.43	5.66E-03
AC008869.1	6.37	6.00E-03
FAM19A2	6.31	1.85E-03
PRKX	6.29	5.10E-03
CSPG4P10	6.26	7.11E-03
INGX	6.22	8.07E-03
MIR3125	6.07	8.32E-03
RNF126	6.06	5.16E-03
AC093763.2	6.05	7.80E-03
AL451042.1	5.97	9.75E-03
AC099548.2	5.97	3.31E-03
PKD1	5.85	5.04E-03
RABEP1	5.80	1.96E-03
OR4A10P	5.64	1.88E-03
ADCY4	5.63	3.08E-03
PLCL1	5.59	5.27E-03
LBX2-AS1	5.59	7.69E-03
AC055717.1	5.47	2.90E-04
CELF6	5.25	7.65E-03
AC092139.3	5.22	8.36E-03
DNAAF2	5.20	7.28E-03
AP001453.4	5.15	6.67E-03
PKLR	5.01	9.04E-03
C16orf54	4.93	1.12E-03
TMEM65	4.85	2.41E-03
ACSL6	4.80	1.17E-03
TMEM241	4.70	4.23E-03
FGD6	4.64	6.16E-03
MPRIP	4.54	1.06E-03
NSUN4	4.49	5.99E-03
PTOV1-AS1	4.42	3.15E-03
AC231981.1	4.41	7.31E-03
ZNF561	4.40	3.09E-03
AL512625.3	4.39	8.25E-03
MACF1	4.30	9.51E-04
IGFBP2	3.88	4.94E-03
AC135050.1	3.55	2.79E-03
AC018716.2	3.11	9.11E-03
FAM160B2	2.83	8.68E-03

B

ANKRD11	-3.72	4.68E-03
MAN1B1	-4.43	5.89E-03
AL162293.1	-4.53	8.28E-03
FZD9	-6.31	4.34E-03
CABP7	-6.54	1.22E-03

Comparison of the most differentially expressed RNAs between samples from patients with RP and positive ANA with samples from patients with RP and negative ANA.
A: Upregulated (highlighted in red).
B: Downregulated (highlighted in blue).

were present in the serum exosomes and were retained following the size selection step in the small RNA library preparation protocol. The different types of RNA molecules contained in the isolated exosomes and their relative proportions are shown in Fig. 1. On average, samples had a low percentage of reads aligned to mature miRNAs (~0.3%), as shown in Figure 1. This is consistent with the relative low abundance of miRNA compared to other RNA types within exosomes. The ENSEMBL biotype annotations were used to assess the distribution of transcript types, based on transcript per million (TPM) values produced by the RSEM-STAR pipeline. A majority of aligned reads originated from protein-coding transcripts, followed by lincRNA, Mt-tRNA and antisense RNA. Even with a relatively low percentage of miRNA alignments from the total sample, a comparison with previously published plasma miRNA datasets confirmed that these SSc samples were representative of plasma exosomes, with a median Pearson's correlation coefficient of R=~0.5.

A review of the most abundant mature miRNAs detected was performed by ranking a total of 403 detected miRNAs by their normalised RPM values, with average RPMs for the top 15 most abundant miRNAs listed in Table II. These values were compared with RPMs reported for blood samples in the extracellular vesicle (EV) miRNA database.

Quantitative miRNA analysis was performed using miRDeep2 mature miRNA normalised RPMs, miRDeep2 mature miRNA read counts, and whole transcriptome read counts produced by RSEM-STAR. A heat map of the differential expression analysis results from the patients with RP with positive ANA in comparison with RP with negative ANA patients is shown in Figure 2 and the actual values obtained are listed in Table III.

Whole-transcript level differential expression was performed on the transcript counts of the two distinct cohorts using the DESeq2 package in R/Bioconductor and transcripts passing a threshold of $p \leq 0.01$. The results are shown in Table IV.

Discussion

The studies described in this report were performed to address the unmet need of identifying non-invasive biomarkers that may allow to distinguish patients with RP not associated to an autoimmune disease from patients with RP that may be at a high risk of evolving into Very Early SSc. The demographic and clinical features of the two cohorts are shown in Table I. The novelty of the approach is that we studied miRNA contained inside circulating exosomes employing unbiased, large scale NGS. Owing to the recent demonstration that miRNAs may play important roles in the pathogenesis of autoimmune diseases including SSc and their particular involvement in the regulation of the molecular mechanisms of tissue fibrosis (15-22), we examined the miRNA levels in this study. The relevance of this work is that it analyses miRNAs contained within exosomes that may be functionally active when cells internalise the exosomes.

The results we obtained corroborated previous studies focused on the analysis of miRNAs contained within serum exosomes as shown in Figure 1 and Table II. The results shown in Figure 2 and Table III indicate that there were six differentially expressed miRNA with high statistical significance (p<0.1) in the comparative analysis between samples of RP with positive ANA patients with samples from patients with RP with negative ANA. The results of a quantitative comparison showed that all the differentially expressed miRNAs were substantially and highly significantly reduced in the samples from RP patients with positive ANA. Some of the differentially expressed miRNAs are detected also in normal human serum exosomes and they show some overlap with miRNA detected in SSc serum exosomes (28).

It must be emphasised, however, that the results obtained here with the unbiased analysis of miRNA contained in serum

exosomes from RP with positive ANA patients that differentiate them from patients with RP with negative ANA will require to be subsequently validated to confirm whether these miRNAs may represent biomarkers to allow the identification of patients with RP at high risk of evolving into SSc. Full validation of these results may also provide further information about the role of specific miRNA in the molecular mechanisms involved in the progression of primary RP into SSc. Most importantly, these results should provide valuable tools that may allow the prompt institution of clinical and therapeutic management of these patients (9-14). The successful identification of these patients may allow prompt therapeutic interventions that should result in a marked reduction of the very high mortality observed in this frequently progressive and often fatal autoimmune disease (33-35).

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