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TITLE: microRNA of Circulating Exosomes for Systemic Sclerosis

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CONTRACTING ORGANIZATION: Thomas Jefferson University, Philadelphia, PA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of the studies performed under the support of this grant was to identify novel and sensitive non-invasive biomarkers for the diagnosis and assessment of the severity and extent of Systemic Sclerosis (SSc) tissue fibrotic involvement. To accomplish this goal we employ a quantitative PCR assessment of the levels of specific miRNAs contained within circulating exosomes isolated from the serum of SSc patients. Three cohorts are included in the study: healthy donors and SSc patients with and without SSc-associated pulmonary fibrosis. We expect that these miRNA biomarkers to be identified in this project may be used as quantitative and unbiased parameters of SSc tissue fibrosis and as indicators for the selection of effective disease treatment based on relevant SSc physiopathologic processes. A total of 142 serum samples from SSc patients and normal subjects that were maintained frozen at the Scleroderma Center serum bank were used to isolate exosomes and the exosome samples were used for further isolation of total RNA, and for synthesizing first strand miRNA cDNA. PCR was performed with 96 cDNA samples and analysis of the results and statistical evaluation are presented.

15. SUBJECT TERMS

Systemic Sclerosis (SSc), biomarkers, serum exosomes, microRNA, SSc-ILD, tissue fibrosis.

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1. INTRODUCTION

Systemic Sclerosis/Scleroderma (SSc) is a serious, disabling, and frequently fatal systemic autoimmune disease of unknown origin characterized by severe vasculopathy and excessive and usually progressive tissue fibrosis affecting the skin and multiple internal organs. The most apparent and almost universal clinical features of SSc are related to the progressive fibrotic process that results in multiple organ failure and a frequently fatal outcome. SSc-associated pulmonary fibrosis (SSc-ILD) occurs in greater than 50% of patients with diffuse SSc and has been recognized as the most important cause of death in SSc. Despite the serious clinical implications of SSc-ILD, well validated biomarkers that allow its early diagnosis and assessment or that carry a predictive prognostic value are not available. This represents a MAJOR UNMET NEED in the management of SSc-ILD causing major delays in the establishment of the clinical diagnosis of SSc-ILD, and therefore, resulting in deleterious delays in the initiation of therapeutic interventions for this highly severe SSc manifestation.

2. KEYWORDS: Systemic Sclerosis (SSc), biomarkers, serum exosomes, microRNA, SSc-ILD, tissue fibrosis.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The main goal of this project is to identify exosome microRNAs that may serve as biomarkers for the diagnosis and assessment of the severity and extent of SSc tissue fibrotic involvement.

The first goal is to examine whether specific exosome miRNAs may allow to differentiate patients with SSc of recent onset from normal individuals and SSc patients with SSc-associated ILD from SSc patients without SSc-associated ILD.

The second goal is to examine whether these exosomal miRNA biomarkers may show a significant change associated with the recurrence of fibrosis following a clinically-relevant therapeutic response in SSc patients who received therapy with an immunomodulatory drug (Mycophenolate).

What was accomplished under these goals?

The goals accomplished to date include the identification and retrieval of serum from the three cohorts of normal and SSc patients. Exosomes were isolated from these sera and following extraction of RNA, cDNAs were synthesized. Real time PCRs assays with oligonucleotides corresponding to specific microRNAs were performed in a substantially large cohort of SSc

patients and every step of the complex procedures for accomplishing the project's goal were optimized.

Exosome solutions were prepared from 142 serum samples (500 μL). RNA was isolated from 99 exosome solutions and polyadenylation and cDNA synthesis were performed from 96 RNA samples. The yield of RNA isolated was an average of approximately 50 ng per sample.

Real time PCR was performed in 96 cDNA samples up to the date of submitting this PROGRESS REPORT. The microRNAs and the sequences used for PCR amplification of each dare listed in Table 1.

Table 1. Sequences of miRNAs.

miRNA	Sequence
hsa-miR-16	TAGCAGCACGTAAAUATTGGCG
hsa-miR-4800-5p	AGTGGACCGAGGAAGGAAGGA
hsa-miR-6126	AGCCTGTGGGAAAGAGAAGAGCAG
hsa-miR-185-5p	TGGAGAGAAAGGCAGTTCCTGA
hsa-miR-4488	AGGGGGCGGGCTCCGGCG
hsa-miR-3196	CGGGGCGGCAGGGGCCTC

Relevant demographic patient information is shown in Table 2.

Table 2. Relevant demographic data of the patients studied.

Cohort	Age (Av)	Gender (%)	SSc Duration (Av/years)
Normal	40	60%F 40%M	
SSc (No ILD)	43	84%F 16%M	4.3
SSc (with ILD)	50	66%F 34%M	4.4

cDNA samples from 43 patients without SSc-ILD, 33 with SSc-ILD and 15 healthy donors were assayed by Agilent PCR System. Differences were calculated employing the comparative Ct method. Figure 1 shows Delta Ct values of five miRNAs.

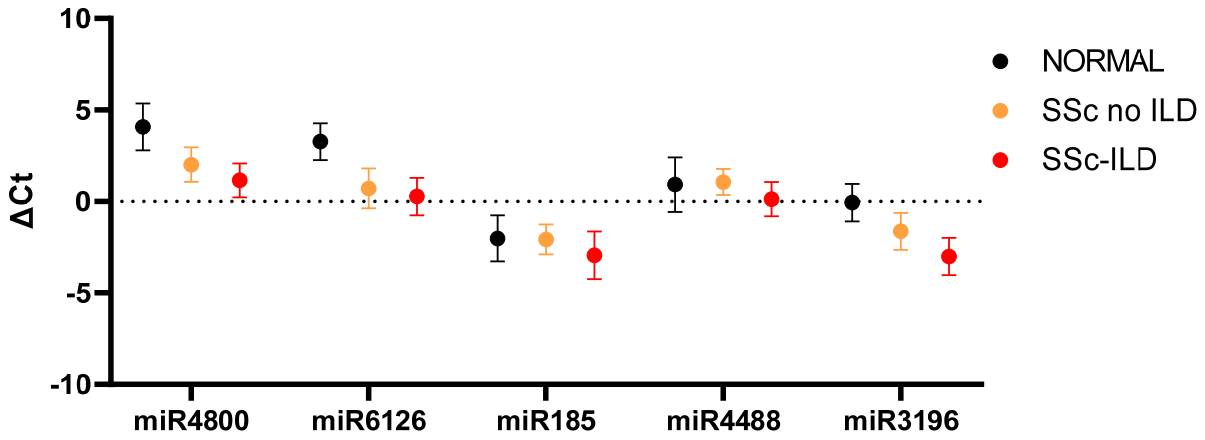

Figure 1. Graph depicting the Delta Ct values for the microRNAs that were analyzed. Values are represented as mean ± 95%CI.

These results demonstrate a differential expression of the miRNA studied, decreasing delta Ct values meaning and increasing expression, therefore an increasing expression of all microRNAs in SSc - ILD vs SSc no ILD and normal samples. Of interest is the statistically significant of the increased expression of miR3196.

Figure 2 shows the qPCR results obtained with 5 miRNAs (miR4800, miR6126, miR185, miR4488 and miR3196). The levels of miR16 were used as a reference gene. The values are shown as the Log2 of the corresponding fold change.

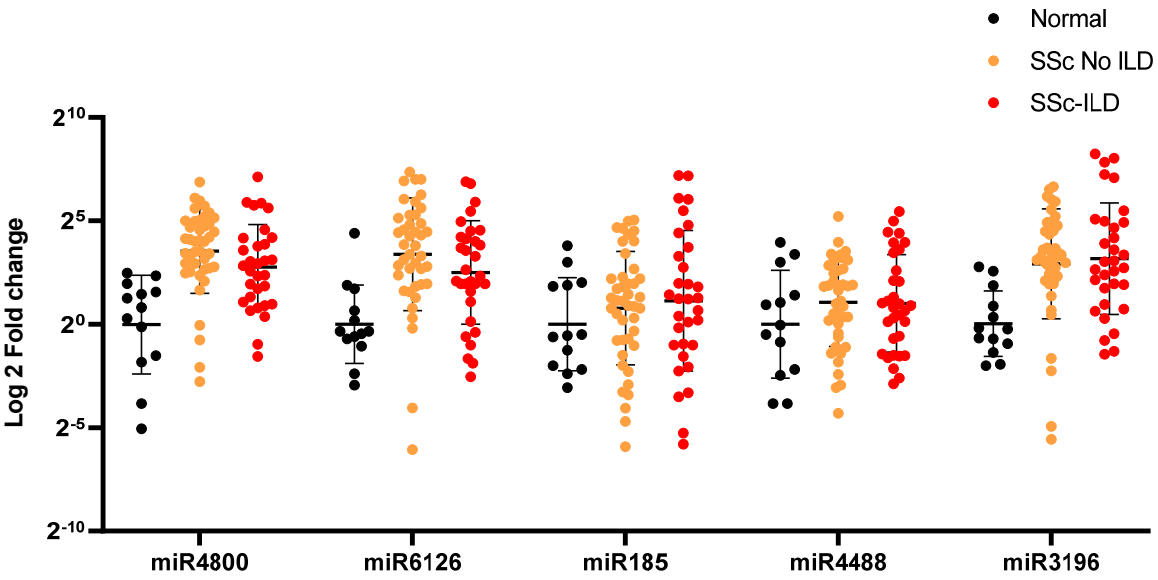

Figure 2. Scatter plots shown the differential expression of miRNA in serum exosomes from SSc patients with and without associated ILD compared to miRNA in serum exosomes from normal or healthy donors. Expression was normalized using miRNA16. Values are represented as mean ± SD.

What opportunities for training and professional development has the project provided?
"Nothing to Report."

How were the results disseminated to communities of interest?
"Nothing to Report."

What do you plan to do during the next reporting period to accomplish the goals?
In order to further accomplish and complete the goals of the project, we will perform the large number of PCRs remaining for all the samples available and employ statistical analysis to identify the specific microRNAs that may serve as biomarkers for the assessment of the severity of the fibrosis in the SSc patients. The specific tasks that will require to be performed include:

1. Performance of assays for 6 miRNAs in a total of 35 serum samples.
2. Repetition of a few experimental assays to confirm and validate the results obtained in the previously performed assays.
3. Performance of statistical analysis of the results to obtain average values, standard deviations and other statistical analysis of all the samples obtained.
4. Establish correlations of the obtained values with the specific Systemic Sclerosis clinical subsets of the patients from whom the serum samples were obtained.
5. Establish correlations of the results obtained with the most relevant Clinical Manifestations and Laboratory Tests and Radiological Studies of the patients that were studied.
6. Establish correlations of the results obtained with the disease duration of the patients included in the study.
7. Prepare the corresponding Data Presentation including Tables and Figures to illustrate the results obtained.
8. Preparation of the Final Report for the Project and of at least one manuscript draft to be submitted for publication to a peer-reviewed scientific journal.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
The potential impact of this study is that it will allow the identification of novel non-invasive biomarkers for SSc-ILD, the fundamental determinant of SSc mortality. The approach proposed

here employing quantitative assessment of the miRNA contained within circulating serum exosomes for biomarker discovery is entirely novel and has not been applied to the field of SSc-ILD. Since it is expected that the project will lead to the identification of non-invasive biomarkers of SSc-ILD that can be used for accurate diagnostic and prognostic assessment of SSc-ILD, the successful outcome of this project will provide substantial advances in the diagnostic process and in the standard of patient care provided to individuals affected by SSc. The identification of such biomarkers would cause a change in the standard of care for SSc-ILD by including an important tool that would be an *in vivo* reflection of the lung fibrotic process in SSc. A further impact of the studies that are being performed is that the results obtained may be of value to indicate the recurrence of SSc-clinical manifestations following discontinuation of a disease-modifying therapeutic intervention, and, therefore, may provide strong support to the re-institution of the therapeutic agent.

What was the impact on other disciplines?
The studies have not yet be completed.
What was the impact on technology transfer?
The studies have not yet be completed.
What was the impact on society beyond science and technology?
"Nothing to Report."

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

No necessary changes were performed.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The initial problems that we encountered in the early stages of this Project were appropriately addressed by performing an improved procedure for exosome isolations from serum and by increasing the sensitivity of the microRNA assays. Therefore new methods for sequencing technics, RNA extraction, First strant-cDNA synthesis and qPCR were performed.

- **Changes that had a significant impact on expenditures**

There were no changes that had a significant impact on expenditures.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None.

6. PRODUCTS: *"Nothing to Report."*

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Sergio A. Jimenez, MD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-5213-1203
Nearest person month worked:	5
Contribution to Project:	Dr. Jimenez has participated in the evaluation of the results obtained with the studies performed. He also assessed the clinical diagnosis and clinical manifestations of the patients from whom the serum samples were analyzed.
Funding Support:	NA

Name:	Sonsoles Piera-Velazquez, MD
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-9121-8026
Nearest person month worked:	11
Contribution to Project:	Dr. Piera-Velazquez has performed essentially all the experimental studies supported by this project and worked with the PI in the interpretation of the results obtained.
Funding Support:	*NA*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS:

NA

9. APPENDICES:

None