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Altered microRNA expression in patients with sarcoidosis

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Abstract. *Background:* Sarcoidosis is a granulomatous multisystem disease of uncertain aetiology. The disease has major inflammatory and immune components; however, the immunopathogenesis is not well understood. Micro ribonucleic acids (microRNAs or miRNAs) are classes of miniature, single-stranded, non-coding RNAs. Their key recognised role includes mediating the silencing of target genes post-transcriptionally. Recently, the role of miRNAs has gained interest in numerous disorders, suggested as being involved in the pathogenesis of those diseases and acting as disease markers. Very little is known about the role of miRNAs in sarcoidosis, with nothing known regarding miRNAs in South African patients, this needs evaluation. The main objective of this research, therefore, was to investigate the serum expression of approximately 800 miRNAs in patients with sarcoidosis compared with race-, age- and gender-matched healthy controls. *Methods:* A total of six patients and six matched controls participated in this study. Whole blood samples were collected in EDTA tubes, processed and the plasma retained. RNA was extracted from the stored plasma samples using the QIAGEN miRNeasy Mini Kit® and concentrated using a salt-ethanol precipitation. The extracted miRNA was profiled using an nCounter® miRNA human v3 expression assay and data analysed using the nSolver™ Analysis Software. *Results:* After excluding one sample/control pair because of cellular RNA contamination, the remaining five patient and five matched control samples were analysed, and 145 miRNAs were found to be potentially differentially expressed. On applying a Bonferroni correction, the only miRNA that was significantly different was miRNA *let-7a-5p*, which was significantly overexpressed (141-fold change; $p<0.0003$) in patients compared with controls. *Conclusion:* This is the first miRNA report of differentially expressed miRNAs in the serum of patients with sarcoidosis and matched healthy controls in South Africa. The results obtained suggest that miRNAs may play a role in sarcoidosis pathogenesis. Whether these molecules have diagnostic or prognostic implications, needs the performance of these tests in future studies recruiting larger patient cohorts.

Key words: sarcoidosis, microRNA, miRNA

Introduction

Sarcoidosis is a multisystem granulomatous disorder of uncertain aetiology. The disease has major inflammatory and immune components; however, the immunopathogenesis is not well understood. Micro ribonucleic acids (microRNAs or miRNAs) are classes of miniature, single-stranded, non-coding RNAs (1). Their key recognised role includes mediating the silencing of target genes post transcriptionally. Recently, the role of miRNAs has drawn attention as both being involved in the pathogenesis, and serving as disease biomarkers, in numerous disorders. Extracellular miRNAs in blood show remarkable stability and resistance to degradation (2,3). MicroRNAs are emerging as biomarkers in many pathophysiological conditions (4) and their role in sarcoidosis

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is only just beginning to be unravelled (5–7). Therefore, the expression of miRNA in sera of sarcoidosis patients was measured and compared with matched controls. The main aim of this study was to examine the serum expression of approximately 800 miRNAs (APPENDIX) in patients with sarcoidosis and race-, age-, and gender-matched healthy controls. Differentially expressed (DE) microRNAs were reported and compared in the two groups.

Patient and methods

Patients and controls

A total of six patients with sarcoidosis and six race-, age- and gender- matched healthy controls were recruited into the study and a variety of demographic, clinical, lung function, laboratory, and radiographic data were collected, where appropriate. The diagnosis of sarcoidosis was made according to the international criteria of the American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and Other Granulomatous Disorders (8). Permission to conduct the study was obtained from the Committee for Research on Human Subjects of the University of the Witwatersrand, and the Hospital, with the requirement for oral informed consent from the patients and controls (M121016 approval).

Study specific investigations

Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and processed within 30 minutes of collection. Tubes were centrifuged for 15 minutes at 3000g, at 4°C, after which the plasma was transferred to a sterile microcentrifuge tube and stored at -70°C until further use.

RNA Extraction

RNA was extracted from the stored plasma samples using the QIAGEN miRNeasy Mini Kit® (Qiagen Sciences, MD, USA). Extractions were performed in duplicate on 200 µl of plasma for each sample. Plasma samples were allowed to thaw completely before commencing with the extractions. Five volumes (1 ml) of QIAzol Lysis reagent were added to each sample and mixed thoroughly. Samples were incubated at room temperature for 5 minutes and 30 attomoles (final concentration) of each of 5 spike-in controls (ath-miR159a, cel-miR-248, cel-miR-254, osa-miR414 and osa-miR442) was added to each extraction and mixed thoroughly. Chloroform at an equal volume to the starting volume (200 µl) was added and the samples shaken vigorously. After incubating at room temperature for 3 minutes, the samples were centrifuged for 15 minutes at 12000g, 4°C, and the upper, aqueous phase transferred to a new tube. One and a half volumes of ethanol were added to each sample, mixed thoroughly and the entire sample loaded onto a RNeasy MinElute spin column. After binding of the RNA to the spin column, the column was washed three times, allowed to dry thoroughly and the RNA eluted from the column twice with 50 µl RNase-free water resulting in a final elution volume of 100 µl per extraction.

Duplicate extractions were then combined, and the RNA concentrated using a salt-ethanol precipitation. The samples were made up to 500 µl with RNAse free water and one tenth the volume (50 µl) DEPC-treated 3M Sodium Acetate was added along with 3 volumes (1.5 ml) ice-cold absolute ethanol. After mixing by inverting the tubes 5 times, the samples were incubated overnight at -20°C. The following day, the RNA was pelleted by centrifugation for 10 minutes at 12000g, 4°C. The resulting RNA pellet was washed twice in ice-cold 70% ethanol and allowed to dry thoroughly before resuspending in 15 µl RNAse free water. Extracts were stored at -70°C until needed.

NanoString analysis

The concentrated RNA extracted from the plasma samples was analysed using an nCounter® miRNA human version 3.0 expression assay according to the manufacturer's protocol. Twelve samples (6 patient samples and 6 age, race and gender matched controls) were analysed on a single cartridge, eliminating the need for calibration between cartridges. Unique oligonucleotide tags, specific for each of the 800 miRNAs in the panel, were ligated to miRNAs from the plasma extracted RNA. These tags allow for increased sensitivity and specificity when detecting the short miRNA sequences. The ligated, tagged miRNAs were denatured at 85°C for 5 minutes, quick-cooled on ice and then hybridised overnight to a capture probe/reporter probe pair. The capture probe has a biotin tag at its 3' end which is

used for binding of the hybridised probes to the imaging surface. The reporter probe has a colour coded molecular barcode at the 5' end which is used to identify the specific miRNA targets during the data collection step.

Following the overnight incubation step, hybridisation mixtures were loaded onto an nCounter® SPRINT cartridge for post-hybridisation processing and analysis with the nCounter® SPRINT analysis system. Within the cartridge, the hybridisation mixtures were purified using a magnetic bead-based methodology to remove excess probe and non-target transcripts. The purified target/probe complexes were then immobilised on the imaging surface using the 3' biotin tags. The unique molecular barcodes on the 5' end of the complexes were scanned and the number of copies of each of the 800 miRNAs counted. These data were extracted to a .csv file and analysed using the nSolver™ Analysis Software, version 3.0. A background subtraction using the geometric mean of the negative controls was included to control for background fluorescence. Counts of more than 10, after background correction, were used to define the presence of a given miRNA. MicroRNA expression levels were normalised between patient and control samples by using the expression levels of the five spike-in controls.

Data analysis and statistics

MicroRNAs with a fold change of less than -1.5 or greater than 1.5, were defined as differentially expressed as fold changes outside this range, are mostly because of natural variation within populations. A Bonferroni correction was applied to correct for multiple comparisons and thus, only miRNAs with p values less than 0.0003 were considered significantly different. Additionally, to account for a high false-positive rate, presumably produced by multiple comparison groups, the p-value adjustment was made using the False Discovery Rate (FDR) process according to Benjamini and Hochberg (9), where the optimal FDR was 5% and a p value ≤ 0.03 was considered to be significant.

Pathway analysis

To evaluate the possible role of the dysregulated miRNAs on gene expression in sarcoidosis, DIANA-miRPath version 3.0 (Deciphering miRNA Function Online software tool) and KEGG (Kyoto Encyclopaedia of Genes and Genomes) was utilised (10). The modified miRNAs' mean fold changes (patients and controls) were used as inputs.

Heat map

The nSolver™ Analysis Software version 3.0 was used to generate the heat map. The heat map of differentially expressed miRNAs are depicted with green colour representing a lower and red colour representing a higher expression level.

Results

The clinical and laboratory parameters of the study patients are shown in Table 1. All were Black patients, 4 males and 2 females, who were non-smokers. The median age of the patients was 47 years, interquartile range (IQR) 39-52 years and minimum and maximum ranges between 30-55 years. Four patients had stage II and two patients had stage IV chest radiographs. Four patients had decreased diffusing capacity for carbon monoxide (DLCO) and three patients had an obstructive defect on spirometry.

When analysing the miRNA results, one patient/control pair was excluded because of cellular RNA contamination in the patient sample. Therefore, only five patients and five matched control samples were analysed. Data of all the microRNAs tested are listed in (APPENDIX). After excluding miRNAs that were below background levels in both the patient and control groups, 145 miRNAs were found to be differentially expressed. Of these, 57 had p-values of less than 0.05 when comparing the patient and control groups, but after applying the Bonferroni correction, (p-values less than 0.0003) only one miRNA, *let-7a-5p* (141.69-fold change, $p < 0.0003$), was significantly overexpressed in patients compared with controls.

Table 2 and Table 3 shows the fold changes of the 15 most over- and under-expressed genes respectively, in the patients with sarcoidosis and Figure 1 shows the expression levels of these 30 genes in the form of a heatmap. Overexpression in sarcoidosis patients is depicted in red and underexpression is depicted in green.

As an alternative to the Bonferroni correction, the Benjamini and Hochberg multiple comparison

Table 1. Clinical characteristics of patients with sarcoidosis and controls

	Patients (n)	(%)	Controls
No. of patients (6 Blacks, all non-smokers)	6		6
Population group – (All Blacks)			
Females	2	(33.3)	2
Males	4	(66.7)	4
Biopsy confirmation	6	(100)	-
Median age (years), (IQR; min-max)	47 (39-52; 30-55)		45.5 (18;26-55)
Median sACE (U/L) (IQR; min-max)	124 (109-219; 92-342)		-
Chest radiograph			
Stage I	0	-	-
Stage II	4	(66.7)	-
Stage III	0	-	-
Stage IV	2	(33.3)	-
Lung function tests			
FVC < 80%	4	(66.7)	-
FEV1 < 80%	4	(66.7)	-
FEV1/FVC <70% *Low DLCO <80%*	3 4	(50.0) (80.0)	- -

Abbreviations: FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; DLCO: diffusion capacity for carbon monoxide; sACE: serum angiotensin-converting enzyme; IQR: interquartile range; Min-max: minimum-maximum; n: number; %: percent

Table 2. The miRNAs with increased expression in patients with sarcoidosis compared with healthy controls

No.	MiRNA	Sarcoidosis	Control	Fold change	p value
1	let-7a-5p	218.39	1.54	141.69	0.0001
2	let-7d-5p	190.55	8.84	21.56	0.0010
3	miR-151a-3p	87.19	4.15	21	0.0038
4	miR-125a-5p	118.56	6.21	19.08	0.0092
5	miR-130a-3p	245.05	16.4	14.95	0.0056
6	miR-361-5p	58.46	4.45	13.15	0.0099
7	miR-148b-3p	31.28	2.78	11.23	0.0041
8	miR-19b-3p	55.11	5.16	10.68	0.0100
9	miR-301a-3p	16.1	1.54	10.44	0.0040
10	miR-664a-3p	21.98	2.13	10.33	0.0045
11	miR-324-5p	15.61	1.54	10.13	0.0136
12	miR-15b-5p	198.62	19.75	10.06	0.0038
13	miR-26a-5p	55.14	5.57	9.90	0.0046
14	miR-199a-3p miR-199b-3p	571.16	57.9	9.86	0.0039
15	miR-181a-5p	192.31	20.19	9.52	0.0025

correction was applied to the 145 potentially differentially expressed miRNAs. Using this method, 12 miRNAs were identified as being significantly different (Table 4), including *let-7a-5p*.

Discussion

It would appear that this is the first report of differentially expressed miRNAs in the serum of

Table 3. The miRNAs with decreased expression in patients with sarcoidosis compared with healthy controls

No.	MiRNA	Sarcoidosis	Control	Fold change	p value
1	miR-3127-5p	2.29	10.2	-4.45	0.0332
2	miR-513c-3p	4.48	15.46	-3.45	0.0653
3	miR-508-3p	4.88	16.2	-3.32	0.0492
4	miR-122-5p	70.72	211.05	-2.98	0.3437
5	miR-103a-3p	3.53	10.17	-2.88	0.1349
6	miR-128-1-5p	9.05	25.41	-2.81	0.1112
7	miR-371a-5p	5.4	14.56	-2.70	0.0990
8	miR-548q	4.71	11.34	-2.40	0.2300
9	miR-498	5.14	12.16	-2.37	0.1449
10	miR-1290	4.66	10.77	-2.31	0.1879
11	miR-549a	10.64	23.6	-2.22	0.0721
12	miR-553	6.85	15.11	-2.21	0.1822
13	miR-520h	14.27	31.57	-2.21	0.1777
14	miR-3136-5p	7.38	16.3	-2.21	0.1624
15	miR-656-3p	8.03	17.58	-2.19	0.0707

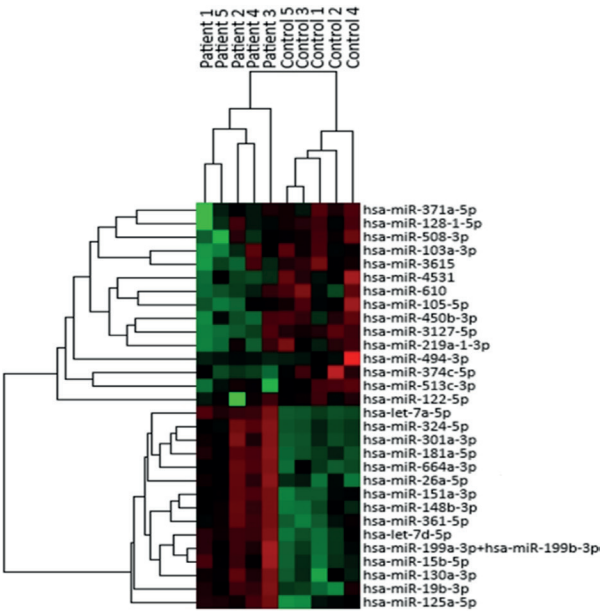

Figure 1. Heatmap of the top 15 most up- and down-regulated miRNAs in patients with sarcoidosis compared with controls. Overexpression in sarcoidosis is depicted in red, and underexpression is depicted in green.

signalling (11). While much research has been dedicated to let-7 target prediction, and to deciphering and understanding its biological function, research into its regulation has only just begun. MicroRNA expression was investigated in the lungs, bronchoalveolar lavage (BAL) fluid, lymph nodes and peripheral blood mononuclear cells (12) and a number of microRNAs seem to play a role in sarcoidosis pathophysiology (13,14). In granuloma formation the TNF-α role has been well documented and is regulated by miRNA let-7 in sarcoidosis (15).

Consistent with the current study, previous studies demonstrated significant differences in miRNA expression between sarcoidosis patients and healthy individuals (5–7,16–19). A previous study conducted by Maertzdorf and colleagues suggested that miRNA-182, miR-355, miR-15b, miR-340 in blood were significantly different between subjects with sarcoidosis and tuberculosis (6). The current study found increased expression of miR-15 in sarcoidosis patients. Fujiwara *et al.*, found the expression of miR-126 and miR-223 significantly higher in sarcoidosis patients than in the healthy individuals in an attempt to determine biomarkers for cardiac sarcoidosis (20). The current study found increased expression of miR-126 in sarcoidosis patients. Novosadova and workers observed dysregulated expressions of miR-146, miR-16, miR-425-5p, and miR-93-5p in all sarcoidosis patients, whether LS was present or not. In patients with LS dysregulated expressions of miR-21-5p and miR-340-5p were found compared to controls and dysregulated expressions of miR-150-5p, miR-1, and miR-212, compared with controls, were found in patients without LS (19). It is worth noting that the differentially expressed miRNAs did not overlap with the differentially expressed

Table 4. The miRNAs that were significantly expressed in patients with sarcoidosis compared with controls (after Hochberg multiple comparison correction)

No.	MiRNA	Sarcoidosis	Control	Fold change	p value
1	let-7a-5p	218.39	1.54	141.69	0.0001
2	miR-1976	1523.68	890.42	1.71	0.0006
3	miR-191-5p	1116.92	122.42	9.12	0.0008
4	let-7d-5p	190.55	8.84	21.56	0.001
5	miR-15a-5p	143.08	22.86	6.26	0.001
6	let-7g-5p	170.77	21.39	7.98	0.002
7	miR-181a-5p	192.31	20.19	9.52	0.002
8	miR-126-3p	393.52	47.28	8.32	0.003
9	miR-151a-3p	87.19	4.15	21	0.003
10	miR-15b-5p	198.62	19.75	10.06	0.003
11	miR-199a-3p miR-199b-3p	571.16	57.90	9.86	0.003
12	miR-301a-3p	16.10	1.54	10.44	0.004

miRNAs in the current study. Asakage and colleagues analysed the dysregulated miRNAs in blood of three major forms of uveitis and identified miR-4323 as the best predictor for sarcoidosis uveitis (21). MiRNAs in sarcoidosis patients with pulmonary fibrosis found that miRNA-222, miRNA-let-7g, miRNA-20a, miRNA-16, miRNA-221, miRNA-126, and miRNA-146a, which inhibit inflammatory response, were all down-regulated in serum samples (22). Again, it should be noted that the decreased expression of miRNAs in this study did not overlap with the down regulated expression of miRNAs in the current study. Similar to the results of the current study, the studies in serum concluded that there were remarkable differences in miRNA levels between sarcoidosis and healthy controls. This difference may be due to the tissue or cycle specificity of the miRNAs, representing the distinct characteristic of disease manifestation between the local tissues and in the general circulation (5).

Bioinformatics web-based computational tools are used to link miRNAs to certain biological processes, dedicated to the evaluation of miRNA regulatory functions and the identification of the pathways involved. DIANA-miRPath version 3.0, Deciphering miRNA Function Online software tool, and KEGG (Kyoto Encyclopaedia of Genes and Genomes) were utilised to determine the potential cumulative impact of the dysregulated miRNAs on sarcoidosis gene expression (10). The fold changes of means (patients and controls) of the modified miRNAs were utilised as inputs. Some of the miRNAs identified as differentially expressed in this study of sarcoidosis, were found to be involved in the "TGF-β signalling pathway" and "Pathways in cancer", amongst others, as has been shown by Novosadova and colleagues (19). Dysregulated expressions of miR-146, miR-16, miR-425-5p, and miR-93-5p were observed in sarcoidosis patients compared with controls and "Pathways in cancer" were predicted by bioinformatics. In addition, between patients with and without Löfgren's syndrome, miR-21-5p, miR-340-5p, and miR-212-3p differed, and their cumulative effect, as was suggested by the current study by different miRNAs, to modulate "TGF-β signalling pathway" (19).

This result can have a profound impact on the regulation and function of immune cell proliferation. It is also impossible to predict the biological effects of altered miRNA expression (5,12,13). MicroRNAs have various probable purposes and can have cumulative consequences when several miRNAs can function on single transcripts (12,13). Each miRNA, therefore, has the potential to control tens to hundreds of genes in several ways, and in most cases several miRNAs affect the expression of the mRNA transcript (16,23).

Basically, the source and function of extracellular circulating miRNA remain unknown (24). There is only a limited understanding of miRNAs and much is yet to be known and understood about the particular and complex function of miRNAs in

sarcoidosis. A solitary miRNA could target several hundred genes and multiple miRNAs can target a solitary gene - determining their exact role, and which miRNA-mRNA sets to focus on, can be perplexing (25). The successful translation of miRNA research is bound to deepen our understanding of sarcoidosis pathogenesis and influence diagnosis, prognosis and treatment. This study provides data showing that plasma miRNA profiles in sarcoidosis can be useful, either as a diagnostic or prognostic biomarker. The intricate interplay between miRNAs and mRNA, predicted target genes by signalling pathways and other components involved in disease pathogenesis and phenotype expression (23), needs further elucidation in a larger cohort.

This study has several limitations. Firstly, the sample size of each group was small. Secondly, not all the components of whole blood which includes immune cells, plasma in which exosomes (small secretory membrane vesicles) from various tissues are present, platelets, and RNA from the gut may contribute to disease pathogenesis. The miRNA expression was a result of all the different cell-types and other components present in whole blood. It would be more relevant to study the immune response in sarcoidosis that may be represented by immune cells (e.g., those in the circulation that would be recruited to tissues), exosomes (derived from diseased tissues) and tissue samples. Thirdly, there is significant clinical phenotypic variation among sarcoidosis patients which might explain why the results of the various cited studies do not align. Future studies should focus on specific disease phenotypes and specific tissue compartments (such as granuloma containing tissues or circulating immune cells) to improve the resolution of the miRNA classifiers. Fourthly, no principal component analysis (PCA) (26) was performed to show that the sarcoidosis patients and controls could be distinguished by the miRNA patterns. However, the miRNA expression patterns and clustering of the samples shown in the heatmap demonstrated clear separation of the case and control groups. Finally, it is conceivable that some miRNAs that may be important for sarcoidosis pathogenesis were not explored in this miRNA panel.

Conclusion

This appears to be the first report of differentially expressed miRNAs in the serum of sarcoidosis patients and matched healthy controls in South Africa. MiRNA *let-7a-5p* was approximately 142 times significantly overexpressed in sarcoidosis patients compared with controls. Many other miRNAs were also either over- or under-expressed. The obtained results suggest that miRNAs play a significant role in the sarcoidosis inflammatory pathogenesis involved in the TGF-β signalling pathway among other pathways. To determine whether these molecules play a pathogenic role in the disease process or could have diagnostic and/or prognostic implications, further studies will be required.

Conflicts of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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