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Regulation of MicroRNA Expression in Scleroderma and Idiopathic Pulmonary Fibrosis: A Research Study

Raveen Badyal, BSc Student [1,2], Beth A. Whalen, BSc [2], Gurpreet K. Singhera, PhD [2,3], Basak Sahin, MSc [2], Kevin J. Keen, PhD [2,3,4]*, Christopher J. Ryerson, MD [2,3], Pearce Wilcox, MD [3], James V. Dunne, MB [3]

[1] Department of Biology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4
[2] Centre for Heart Lung Innovation, Providence Research and University of British Columbia, Vancouver, British Columbia, Canada V6Z 1Y6
[3] Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3
[4] Department of Mathematics and Statistics, University of Northern British Columbia, British Columbia, Canada V2N 4Z9

*Corresponding Author: kevin.keen@hli.ubc.ca

Abstract

Introduction: Scleroderma (SSc) is an autoimmune disorder with the hallmark of fibrosis of the skin, vasculature and internal organs. Patients with SSc and undifferentiated connective tissue disease (UCTD) are susceptible to interstitial lung disease (ILD), leading to decreased lung function and death. Idiopathic pulmonary fibrosis (IPF) is a form of ILD that is not associated with extrapulmonary manifestations. In this study, lung involvement of SSc was studied by observing how disease progression and pathogenesis differ among patients with SSc, UCTD, and ILD compared to healthy controls and patients with IPF. Our group has previously identified disease targets through microRNA sequencing, including the DICER enzyme, which works closely with the protein DGCR8 and the enzyme DROSHA in the RNA interference pathway. The canonical pathway stipulates that DICER processes microRNAs in the cytosol while DGCR8 and DROSHA process microRNAs in the nucleus. DICER, DROSHA, and DGCR8 are hypothesized to contribute to ILD progression.

Methods: Human peripheral blood mononuclear cells (PBMCs) were isolated from voluntary participants, including healthy controls. PBMCs were subsequently lysed with subcellular fractionation buffer. Western blotting was done on the resulting cytosolic and nucleic fractions for DICER, DROSHA, and DGCR8 protein expression. The cytosolic fractions were normalized to GAPDH, while the nucleic fractions were normalized to B2M. Nonparametric Kruskal-Wallis tests were used for statistical analysis.

Results: The medians were significantly higher for healthy controls for DICER in the nucleus with a p-value of 0.0302, and DROSHA in the cytosol with a p-value of 0.0406 compared to patients with SSc, UCTD, and IPF.

Discussion: Differences in expression were found for DROSHA in the cytosol and DICER in the nucleus, suggesting dysregulation of the non-canonical RNA interference pathways in SSc, UCTD, and IPF patients. Variability of disease progression within the groups could lead to variable enzyme and protein levels within the same disease status. With larger sample sizes, statistically insignificant differences would become significant. Lipid nanoparticle technology could be used to deliver deficient microRNAs to silence mRNA in patients.

Conclusion: Due to dysregulation of the RNA interference pathway, microRNAs may be inadequately processed in the patient groups.

Keywords: scleroderma; systemic sclerosis; RNA interference; idiopathic pulmonary fibrosis; microRNA; peripheral blood mononuclear cells; western blot; DICER; DROSHA; DGCR8

Introduction

Systemic sclerosis (SSc), also known as scleroderma, is a chronic multisystem autoimmune disorder characterized by skin, vascular and organ fibrosis. Hallmarks of SSc are vasculopathy, excess collagen production, fibroblast dysfunction, excess production of autoantibodies and cytokines, and increased skin-cell turnover. The biomolecular mechanisms underlying systemic sclerosis are not fully understood. There may be multiple causes [1]. Several genes are associated with the development of SSc, such as the human leukocyte antigen genes, but the effect of these loci is low [2]. Further, epigenetic dysregulation also appears to be a prominent feature of SSc [2]. Environmental factors, which may trigger epigenetic pathways, could also be involved. These environmental factors include silica dust, organic solvents, cytomegalovirus, Epstein-Barr virus and

parvovirus B19 [3-6]. However, the mechanisms underlying the response to environmental factors are still unclear.

Systemic sclerosis can be differentiated into limited cutaneous (lcSSc) and diffuse cutaneous forms (dcSSc) [7] according to the extent of skin damage. For the limited cutaneous form, skin involvement affects the face but is distal to the elbows and knees, while the diffuse cutaneous can affect the entire body [8]. SSc has an estimated prevalence of 44 per 100,000 in Canada and is more common in females than males [9].

The age of onset of SSc varies but is most common in middle age with one quarter of SSc patients presenting at age 60 years or above with variable severity [10]. The onset of dcSSc is generally younger compared to lcSSc [11]. Increased involvement of the lung and heart may occur in older patients [10]. A meta-analysis of standardized mortality ratios in SSc patients found that the leading causes of death in SSc are interstitial lung disease (ILD) and pulmonary arterial hypertension. Overall, older age at onset has a worse prognosis [12].

As a systemic disease, involvement is beyond the skin and may include the gastrointestinal tract, kidneys, heart, and lungs [13]. Scleroderma patients also experience Raynaud's phenomenon, which involves reduced circulation to the fingers and other body parts due to blood vessel abnormalities [14]. The 2013 Classification Criteria for Systemic Sclerosis specifies that "skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints," which is also classified as the first non-Raynaud's phenomenon, is a sufficient criterion for an SSc diagnosis [15]. Lung involvement can progress to ILD, with the most common pattern being nonspecific interstitial pneumonia (NSIP) which occurs in approximately 65% of cases and is characterized by peripheral ground-glass opacities with an apical to basal gradient, associated reticulation, and bronchiectasis [16]. In contrast, the usual interstitial pneumonia pattern (UIP) occurs in approximately 25% of patients with SSc-ILD, but is the principal pattern found in patients with idiopathic pulmonary fibrosis (IPF) [16,17]. Foci of fibroblastic activity and honeycomb change are hallmarks of the UIP pattern. UIP is generally associated with a more rapid progression than NSIP [16,17]. The typical first sign of lung damage is difficulty breathing on exertion, and this is confirmed by lung function tests and CT imaging [18]. There are pharmacological interventions for lung damage. These include immunosuppressants such as cyclophosphamide, mycophenolate, and tocilizumab, which stabilise fibrosis [19,20]. When immunosuppressants are used in combination with nintedanib, which is a receptor blocker for multiple tyrosine kinases, elaboration of fibrogenic growth factors is diminished [19-21]. The anti-fibrotic agent pirfenidone is used to treat IPF patients and is being trialed in SSc. Pirfenidone inhibits transforming growth factor-β stimulated collagen synthesis, which decreases accumulation of the extracellular matrix and limits fibrosis [22]. End-stage treatments for ILD in SSc are either bilateral lung transplantation or palliative care with supplemental oxygen [23].

IPF is of unknown cause and is characterized by progressive fibrosis and scarring in the lungs [24]. IPF is estimated to have a prevalence of 12 per 100,000, and it is more common in males [25,26]. Both pirfenidone and nintedanib have been demonstrated in clinical trials of IPF patients to slow the rate of decline of predicted forced vital capacity and may yet be shown to reverse fibrosis [22,27].

Undifferentiated connective tissue disease (UCTD) has an autoimmune profile that does not fulfill the diagnostic criteria for systemic sclerosis or other CTDs such as lupus or dermatomyositis. Treatment for UCTD-ILD parallels that for SSc-ILD.

Disrupted microRNA levels are thought to be involved in the mechanism driving SSc [28,29]. The enzyme DICER, which is encoded by the DICER1 gene, is a part of the RNA interference pathway, which can result in post-transcription gene silencing. In the canonical pathway, DICER processes microRNAs in the cytosol. The protein DiGeorge syndrome critical region 8 (DGCR8) and enzyme DROSHA are part of the microprocessor complex of this pathway, which, according to the canonical pathway, processes microRNAs in the nucleus. Nevertheless, non-canonical functions may also exist for DICER, DGCR8, and DROSHA. In the nucleus after transcription, the primary microRNA is cleaved by the microprocessor complex [30]. The resulting pre-microRNA is transported to the cytosol, where it is cleaved by the endonuclease DICER to form mature microRNA that can be incorporated into the RNA-induced silencing complex (RISC) [30]. The RISC then binds and cleaves mRNA, destabilizing it, which leads to gene silencing as translation is inhibited [31]. Through exploratory work to determine whether DICER has a role in the pathophysiology of SSc, real-time qPCR showed significant differences in mRNA expression among healthy controls and the disease groups. To discover whether there may be a dysregulation in this pathway, DGCR8 and DROSHA mRNA expression were measured along with DICER. To validate these results, protein expression for each of these three targets was determined through western blot analysis.

Methods

To determine the possible roles of DICER, DROSHA and DGCR8 in the pathophysiology of SSc, UCTD, and IPF, whole blood was collected with informed consent from normal controls and patients with SSc, UCTD, or IPF—in accordance with harmonized research ethics approval by the University of British Columbia Providence Health Care Research Ethics Board and the University of Northern British Columbia Research Ethics Board.

Cell Isolation

Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were isolated from 20-30 milliliters of whole blood collected in Heparinized Vacutainers (BD Biosciences). White blood cells were separated by density gradient centrifugation with density gradient mediums such as Lymphoprep (Stemcell Technologies) for isolation of PBMCs or Histopaque-1077 (Sigma-Aldrich) and Histopaque-1119 (Sigma-Aldrich) for isolation of both PBMCs and PMNs. PBMCs and PMNs were lysed with a subcellular fractionation buffer (buffer (0.35 M EGTA, 1 M KCl, 0.5 M MgCl2, 0.25 M EDTA, 1 M HEPES, and 1 M DTT) to collect total protein lysates.

Cell Fraction Lysis

Isolated PBMCs and PMNs were suspended in Gibco Dulbecco's phosphate-buffered saline (DPBS). The cell pellets were then resuspended in 200 µL of subcellular fractionation buffer containing an EDTA-free protease inhibitor cocktail (MilliporeSigma) and PhosSTOP phosphatase inhibitors (MilliporeSigma). Resuspended cells were fractionated by the shearing force of a 1 mL 25G syringe (BD Biosciences). After centrifugation, the cell-free supernatant was saved as cytosolic protein, whereas the cell pellet was resuspended in DPBS as the nucleic protein. Samples were stored in a -80ºC freezer until further use.

Western Blot

Total protein concentrations of the cell fractions were quantified colorimetrically using the Pierce Bicinchoninic Acid (BCA) protein assay as per the manufacturer's protocol (Thermo Scientific).

To detect B2M, GAPDH, DGCR8, DROSHA, and DICER: 30 µg of the nucleic fraction and 15 µg of the cytosolic fraction were prepared with a 1x sample buffer containing β-mercaptoethanol. Initially, all protein targets, including DICER (217 kDa), DROSHA (157 kDa), DGCR8 (110 kDa), GAPDH (37 kDa), and B2M (11 kDa) were run on a 4-15% Mini-PROTEAN TGX Precast Protein Gels (BioRad). However, to ensure that there was optimal separation between the higher weight proteins, B2M was run off of the gels. B2M expression was obtained through a second run of western blots due to its low molecular weight.

The prepared samples were separated on 4-15% gels, or 15% SDS-Page gels for B2M, for adequate separation between the targets. The Spectra Multicolor High Range Protein Ladder (Thermofisher) and ColorMixed Protein Marker 180 (Abclonal) were used to capture the proteins of interest. The gel was then transferred onto a nitrocellulose membrane using transfer buffer containing 10% Methanol. The membranes were then blocked in 5% skim milk in TBST (10 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween-20). The blots were then incubated overnight at 4 C with their respective primary antibodies from Abcam at the following dilutions: DGCR8 at 1:5000, DROSHA at 1:25000, and DICER at 1:2000 and from Abclonal: B2M at 1:1000. After overnight incubation with primary antibodies, the membranes were washed with TBST, then probed with the respective secondary antibodies. The B2M, DGCR8, and DROSHA blots were probed with secondary goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated antibody at 1:2000 dilution (MilliporeSigma). The DICER blot was probed with the secondary goat anti-mouse IgG HRP at 1:2000 dilution (BD Biosciences). The GAPDH blot was probed with HRP-conjugated GAPDH antibody (Cell Signaling Technology) at 1:1000 dilution.

After incubation of the blots with HRP labelled antibodies, the blots were washed in TBST. This was followed by the utilization of the SuperSignal™ West Femto Maximum Sensitivity ECL Substrate (Thermo Scientific) to detect protein expression for densitometry analysis using the Genesys software with the G:BOX system to capture images.

The nucleic samples were normalized to B2M, and the cytosolic samples were normalized to GAPDH for comparing densitometry. Densitometry was done on the ImageJ image processing and analysis in Java software (National Institutes of Health) to obtain semi-quantitative data for statistical analysis.

Statistical Analysis

After data collection, nonparametric Kruskal-Wallis tests were done with GraphPad Prism 5 (GraphPad Software Inc., California, USA) software to determine whether there were differences among the locations of the distributions of the seven groups. Fisher's exact test was done to assess differences in frequencies for categorical variables. The cut-off p-value to determine the significance of this exploratory work was set to 0.05. The GraphPad Prism 5 software was used to generate the graphs for the protein expression results.

Results

Participant Demographics

The characteristics of the participants are given in Table 1 along with p-values for tests of no differences among groups. With a cut-off p-value of 0.05, the variables with significant differences are age and disease duration at date of phlebotomy for the western-blot samples, percent-predicted forced vital capacity (FVC) and diffusing level of carbon monoxide (DLCO). Note that the patients with IPF are older with disease duration shorter compared to the patients with a connective tissue disease. Those patients with ILD have lower percentage FVC and DLCO compared to patients with SSc without ILD as expected.

Table 1. Participant demographics

Disease Status	Control	IPF	lcSSc No ILD	lcSSc ILD	dcSSc No ILD	dcSSc ILD	UCTD ILD	P-value
Count	7	9	5	12	6	12	4	N/A
Proportion Male (%)	28.6	66.7	0.0	33.3	16.7	41.7	25.0	0.2675
Median Age (year)	48	70	68	59	55	54	62	0.0024
Disease Duration (year)	N/A	0.83	15.0	6.0	5.5	5.5	3.0	0.0022
Treatment Presence Count (%)	N/A	66.7	20.0	91.7	66.7	83.3	75.0	0.0686
Ever Smoker Count (%)	0.0	66.7	20.0	25.0	50.0	33.3	75.0	0.1003
Median Baseline FVC %	N/A	82.0	108.0	71.0	101.0	65.0	60.5	0.0396
Median Baseline DLCO %	N/A	46.0	81.0	64.5	83.0	56.0	44.5	0.0323

Notes: Disease duration is determined from date of ILD first seen on high-resolution computed tomography in IPF and UCTD ILD but time from first non-Raynaud's phenomenon in patients with SSc. Treatment includes presence of ILD therapies: nintedanib, pirfenidone, mycophenolate mofetil, azathioprine, tocilizumab, rituximab, and cyclophosphamide. P-values for the categorical variables and the continuous variables are by Fisher's exact test for frequencies and the Kruskal-Wallis test, respectively.

Abbreviations: FVC: forced vital capacity; DLCO: diffusing level of carbon monoxide; N/A: not applicable

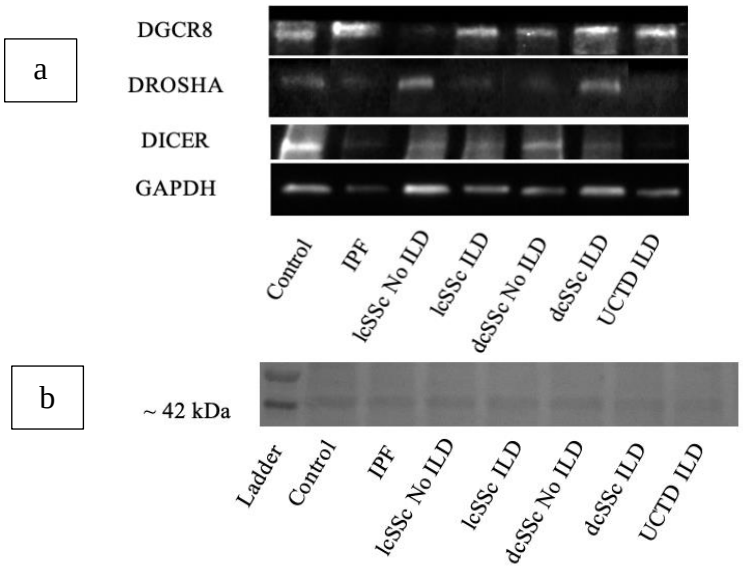


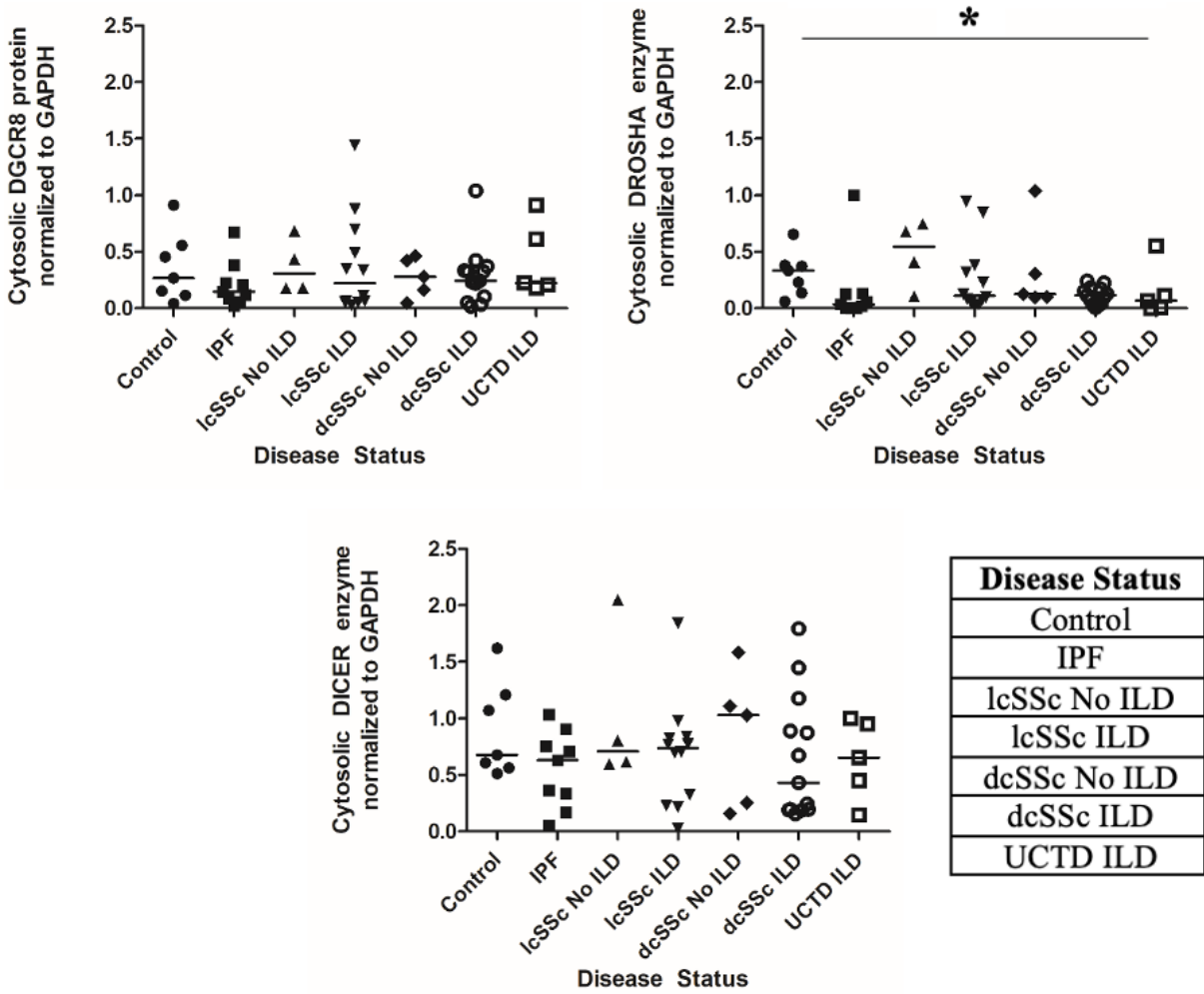
Figure 1. (a) Representative western blot images of DGCR8, DROSHA, DICER, and GAPDH protein expression in the cytosolic fraction of PBMCs. (b) A Ponceau stain of the housekeeping protein β-actin (~ 42 kDa) from the cytosolic fraction of PBMCs. Genesys software was used with the G:BOX system to capture images.

PMN

After multiple rounds of optimization for PMNs to detect DGCR8, DROSHA, and DICER protein expression via western blot, a signal could not be detected in PMNs with the attempted methods. The optimized cell isolation and lysis methods were then applied to PBMCs only.

DGCR8, DROSHA and DICER in the Cytosol

The western blots in Figure 1a illustrate the relative expressions of DGCR8, DROSHA, DICER, and GAPDH in the cytosolic fraction for one representative member in each group. A Ponceau stain of the cytosolic proteins in Figure 1b confirms equal loading of protein as β-actin expression is consistent between the samples. Each member was chosen randomly from each group. The images in Figure 1 are representative of the overall values demonstrated by densitometry. DGCR8 in the cytosol normalized to GAPDH tends to be higher in patients and the controls who do not have ILD or IPF (Figure 2), but the differences among the groups are not statistically significant (p-value: 0.8395). Evidence for differences in location in cytosolic DROSHA normalized to GAPDH for the seven participant categories was stronger (p-value: 0.0406). The medians depicted in Figure 2 for cytosolic DROSHA for controls and participants with lcSSc without ILD appear to be higher than participants with ILD or IPF. Participants with dcSSc without ILD appear to have slightly higher levels of cytostolic DROSHA compared to participants with ILD. Differences in location for the seven categories of participants for DICER in the cytosol normalized to GAPDH was not statistically significant (p-value: 0.7975).



Disease Status	n
Control	7
IPF	9
lcSSc No ILD	5
lcSSc ILD	12
dcSSc No ILD	6
dcSSc ILD	12
UCTD ILD	4

Figure 2. Protein expression of DGCR8, DROSHA, and DICER in the cytosol normalized to GAPDH determined via western blot analysis. Horizontal line segments denote medians. The * symbol denotes a significant difference among the medians of the groups. The p-value for DROSHA in the cytosol normalized to GAPDH is 0.0406. GraphPad Prism 5 software was used to generate the graphs.

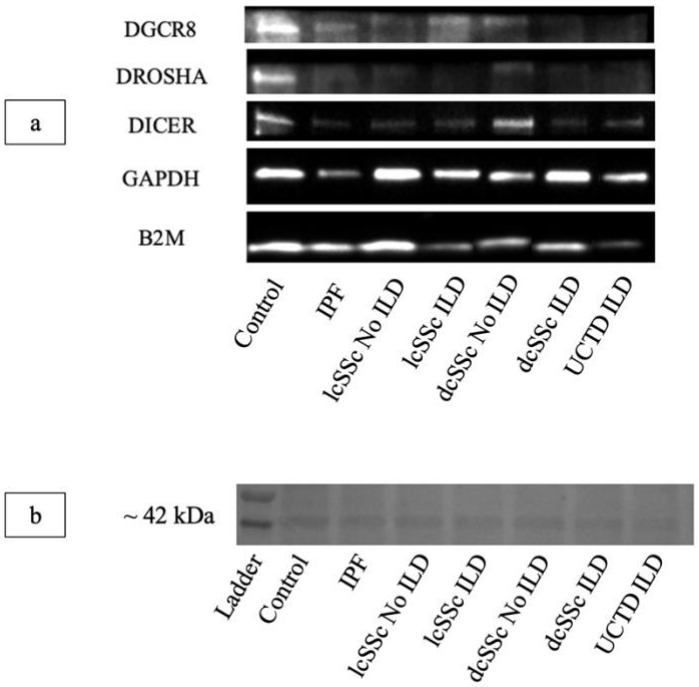
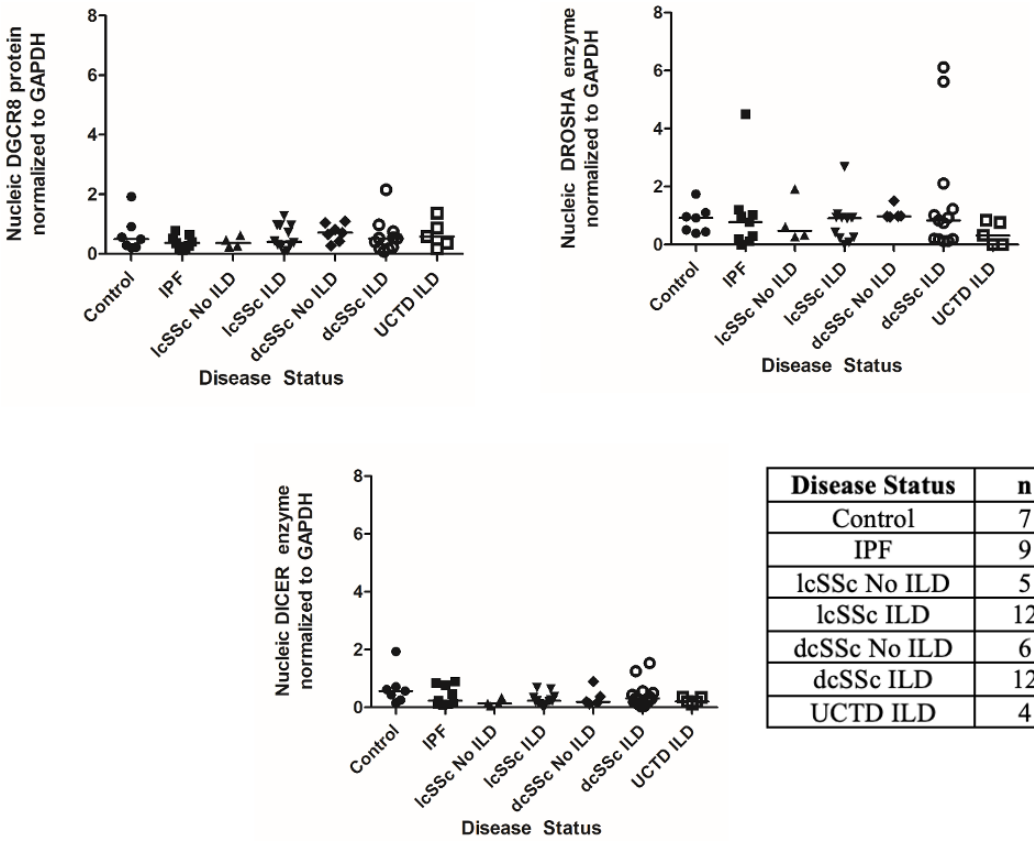


Figure 3. (a) Western blot images of DGCR8, DROSHA, DICER, GAPDH, and B2M protein expression in the nucleic fraction of PBMCs. (b) A Ponceau stain of the housekeeping protein β-actin (~42 kDa) from the nucleic fraction of PBMCs. Genesys software was used with the G:BOX system to capture images.



Disease Status	n
Control	7
IPF	9
lcSSc No ILD	5
lcSSc ILD	12
dcSSc No ILD	6
dcSSc ILD	12
UCTD ILD	4

Figure 4. Protein expression of DGCR8, DROSHA, and DICER in the nucleus normalized to GAPDH determined via western blot analysis. Horizontal line segments denote medians. GraphPad Prism 5 software was used to generate the graphs.

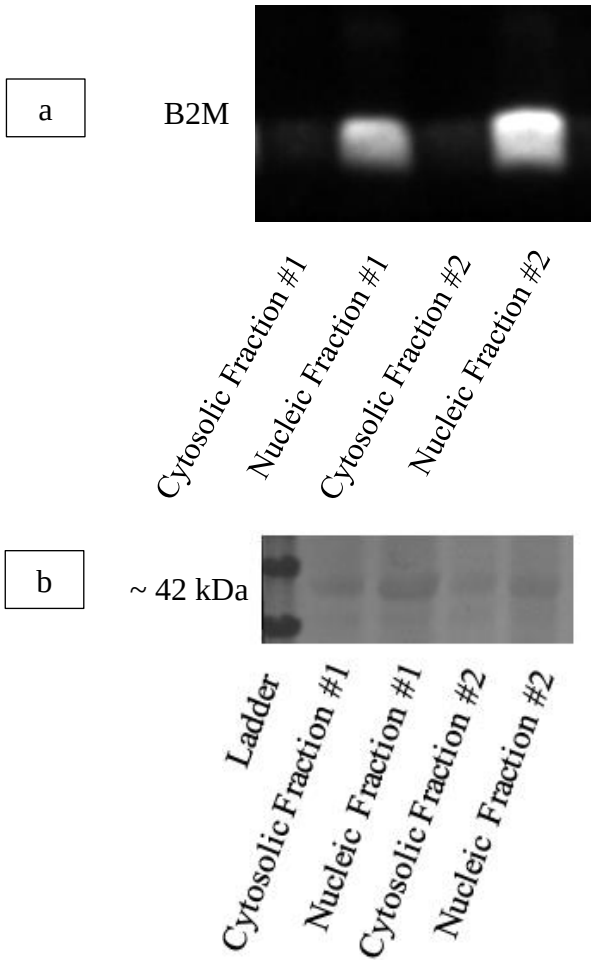


Figure 5. (a) Western blot image of B2M protein expression in nucleic and cytosolic fractions of PBMCs. (b) A Ponceau stain of the housekeeping protein β-actin (~42 kDa) from cytosolic and nucleic fractions of PBMCs. Genesys software was used with the G:BOX system to capture images.

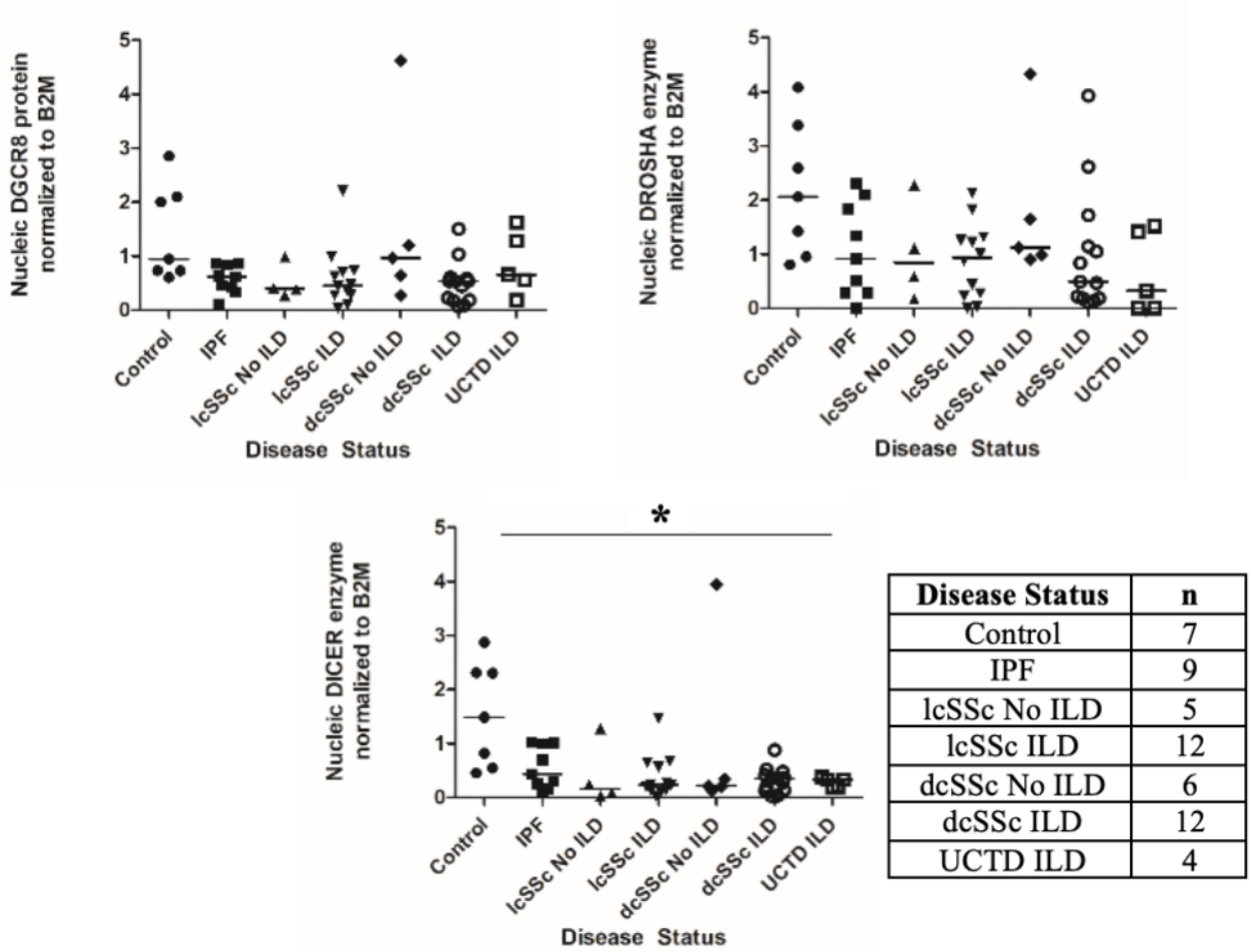
DGCR8, DROSHA and DICER in the Nucleus Normalized to GAPDH

Expression of DGCR8, DROSHA, DICER, GAPDH, and B2M are depicted for a representative member of each group in Figure 3a. Each member is the same as the member randomly selected from each group in Figure 1. A Ponceau stain of the nucleic proteins in Figure 3b confirms equal loading of protein, as β-actin expression is consistent between the samples. When normalizing the nucleic fractions to GAPDH, the Kruskal-Wallis test among the seven groups for each of the three targets was not statistically significant (Figure 4).

DGCR8, DROSHA and DICER in the Nucleus Normalized to B2M

While GAPDH was observed at similar amounts in both cytosolic and nuclear fractions, B2M expression was visibly higher in the nucleus (Figure 5a) so it was decided that B2M was a better housekeeping target for the nuclear fraction samples due to B2M's presence in nucleated cells. The Ponceau stain image in Figure 5b shows even loading of the nucleic and cytosolic samples, which confirms that the difference observed in B2M expression is due to B2M's nuclear localization.

In Figure 6, evidence for differences in DGCR8 in the nucleus normalized to B2M among the seven categories of participants was weak (p-value: 0.0541). Evidence for differences in DROSHA in the nucleus normalized to B2M among the seven categories of participants was weaker (p-value: 0.1862). Levels of DGCR8 and DROSHA in the nucleus in either controls or dcSSc participants without ILD appeared to be higher compared to the other groups. Evidence for differences in location among the participant categories for nucleic DICER enzyme expression normalized to B2M was stronger (p-value: 0.0302). Levels of DICER in the nucleus appeared higher for healthy controls compared to the other categories.



Disease Status	n
Control	7
IPF	9
lcSSc No ILD	5
lcSSc ILD	12
dcSSc No ILD	6
dcSSc ILD	12
UCTD ILD	4

Figure 6. Protein expression of DGCR8, DROSHA, and DICER in the nucleus normalized to B2M determined via western blot analysis. The * symbol denotes a significant difference among the medians of the groups. The p-value for DICER in the nucleus normalized to B2M is 0.0302. GraphPad Prism 5 software was used to generate the graphs.

Discussion

In the canonical RNA interference pathway, DICER is localized to the cytosol, and DGCR8 and DROSHA are localized in the nucleus. Nevertheless, these targets do exist outside of these areas in mammalian cells and can process microRNAs in a non-canonical manner [32,33]. The significant differences among our groups were for DROSHA in the cytosol and DICER in the nucleus, which is evidence for non-canonical pathways being responsible for the differences among our groups.

Multiple attempts to detect the protein expression of the three targets in PMNs failed. To ensure that the standard lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/mL leupeptin) from Cell Signalling Technology was not a limiting factor in the release of the proteins from the PMNs, different buffers were used, including a subcellular fractionation and a RIPA buffer (Sigma-Aldrich). Further, a water bath sonication was attempted, but still no signal was detected. It could be that PMNs do not have a sufficient concentration of these targets, or the neutrophils were becoming activated during the isolation and lysis process, and this reduced the number of neutrophils [34]. The signal in PBMCs however was robust.

Further, as per standard practice, B2M should have been run on the blots with GAPDH, DGCR8, DROSHA and DICER. The proteins of interest had a wide range of molecular weights as the lowest weight protein was B2M at 11 kDa and the highest weight protein was DICER at 217 kDa. One of the optimization attempts was to run all the proteins on the same gel, but there was not an adequate separation between the proteins with higher molecular weight despite using gradient gels. Due to this limitation, B2M expression was obtained through a second run of western blots.

There were two outliers in our data for DROSHA in the nucleus. The two outliers that had a higher amount of DROSHA in the dcSSc group were males. In males, there is a more severe and rapid onset of disease. We may be

observing differences in protein expression due to the differences between disease pathogenesis and progression of SSc between males and females. Additionally, these two patients were also exposed to tobacco and silica dust, which are also factors in exacerbating disease. Further work to understand the differences in the microRNA processing pathway between males and females with SSc is required to better understand the outliers in our dataset.

As the statistical analysis for DGCR8 in the nucleus normalized to B2M resulted in a p-value of 0.0541, the null hypothesis can not be rejected as this value is greater than 0.05. If the magnitude of the p-value is considered, then a p-value of 0.0541 suggests weak evidence for a difference compared to the results for DROSHA and DICER which had p-values of 0.0406 and 0.0302 respectively. For a sufficiently large sample size, a real difference in DGCR8 protein expression analysis would yield a p-value less than 0.05 according to the strong law of large numbers.

While statistically significant differences among the participant categories in the canonical pathway were not found, the expression of the enzymes DROSHA and DICER is higher in healthy controls and patients with SSc without ILD. As SSc progresses with time, either skin or lung damage, or both, typically increase in severity [35]. Other factors such as age, gender, and smoking status could be responsible for differences in DGCR8, DROSHA, and DICER expression between controls and patients of the same disease status [36-38].

Conclusions

In this proof-of-concept study, there are significant differences between DROSHA in the cytosol and DICER in the nucleus suggesting that non-canonical RNA interference pathways appear to be dysregulated in SSc, UCTD, and IPF patients. Variability of disease activity, progression, and severity within the groups likely leads to variable enzyme and protein levels leading to variability in enzyme function.

Confirming these results will require a larger sample of progressive versus non-progressive SSc and UCTD, and a time series of enzyme amount and activity. Similarly, in IPF, the difference between males and females, early and late disease, and smoker and non-smoker need to be addressed.

There are presently limited pharmacological options for pulmonary fibrosis. In the future, lipid nanoparticle technology could deliver microRNAs to silence the mRNAs driving excess collagen deposition and fibroblast dysfunction to forestall bilateral lung transplantation or palliative care in end-stage disease.

With significant differences found in the expression of these proteins and enzymes of the RNA interference pathway, it is possible that one or more microRNAs are not being processed correctly. Proteins that should be silenced are being expressed abnormally and could be causing increased deposits of collagen in tissues. Deficient microRNA could be supplemented orally or via injection. Nanoparticle technology could deliver the microRNAs to the cytosol or nucleus effectively as lipid nanoparticles can effectively penetrate the lipid bilayer of cell membranes.

List of Abbreviations Used

SSc: systemic sclerosis
lcSSc: limited cutaneous systemic sclerosis
dcSSc: diffuse cutaneous systemic sclerosis
ILD: interstitial lung disease
NSIP: nonspecific interstitial pneumonia
UIP: usual interstitial pneumonia
IPF: idiopathic pulmonary fibrosis
UCTD: undifferentiated connective tissue disease
DGCR8: DiGeorge syndrome critical region 8
RISC: RNA-induced silencing complex
PBMC: peripheral blood mononuclear cell
PMN: polymorphonuclear cells
DPBS: Dulbecco's phosphate-buffered saline
BCA: bicinchoninic acid
HRP: horseradish peroxidase
B2M: β-2 microglobulin
FVC: forced vital capacity
DLCO: diffusing level of carbon monoxide
N/A: not applicable

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

All study participants were volunteers, and each gave written informed consent in accordance with harmonized research ethics approval by the University of British Columbia Providence Health Care Research Ethics Board and the University of Northern British Columbia Research Ethics Board.

Authors' Contributions

RB: assisted with project design and planning, collected and analysed data, drafted the manuscript, and gave final approval of the version to be published.
BAW: made substantial contributions to the design and acquisition of the work, reviewed the manuscript critically and gave final approval of the version to be published.
GKS: assisted with project design and planning, experimental troubleshooting, manuscript editing, and gave final approval of the version to be published.
BS: contributed to the design of the study, assisted in revision of the manuscript and performed previous assays for the project that identified targets for this study.
KJK: made contributions to the design, analysed data, assisted in the drafting and editing of the manuscript, and gave final approval of the version to be published.
CJR: contributed to cohort generation and data acquisition, interpretation of results, production of the final manuscript, and gave final approval of the version to be published.

PW: contributed to the design and planning of the study, manuscript editing, and gave final approval of the version to be published.
JVD: contributed to the design and planning of the study, manuscript editing, and gave final approval of the version to be published.

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