Prediction of severity and subtype of fibrosing disease using model informed by inflammation and extracellular matrix gene index

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Abstract

Fibrosis is a chronic disease with heterogeneous clinical presentation, rate of progression, and occurrence of comorbidities. Systemic sclerosis (scleroderma, SSc) is a rare rheumatic autoimmune disease that encompasses several aspects of fibrosis, including highly variable fibrotic manifestation and rate of progression. The development of effective treatments is limited by these variabilities. The fibrotic response is characterized by both chronic inflammation and extracellular remodeling. Therefore, there is a need for improved understanding of which inflammation-related genes contribute to the ongoing turnover of extracellular matrix that accompanies disease. We have developed a multi-tiered method using Naïve Bayes modeling that is capable of predicting level of disease and clinical assessment of patients based on expression of a curated 60-gene panel that profiles inflammation and extracellular matrix production in the fibrotic disease state. Our novel modeling design, incorporating global and parametric-based methods, was highly accurate in distinguishing between severity groups, highlighting the importance of these genes in disease. We refined this gene set to a 12-gene index that can accurately identify SSc patient disease state subsets and informs knowledge of the central regulatory pathways in disease progression.

Introduction

Fibrosis results from continuous connective tissue remodeling during a reparative or reactive process, leading to disrupted tissue function in affected organs. The high mortality rate from fibrosing diseases is a multifaceted health issue in the developed world [1] that continues to demand further exploitation. Progress in this area requires reverse translation of clinical findings that inform preclinical studies, and re-validation and/or generation of existing or new animal models.
Fundamental to the challenges in generating effective treatments for the majority of patients is the heterogeneity of fibrosing diseases’ symptom patterns, progression, and severity. Current research has focused on the causes of fibrosis, the discovery of fibrosis-associated biomarkers, and the associations between fibrosis and disease [2–5]. Further inquiry is needed to gain a deeper understanding of progression of the fibrosing state. Notably, addressing the heterogeneity of fibrosing diseases is essential in providing a clear link between the multifaceted genomic and phenotypic changes of fibrosis.

Promisingly, new high-throughput ‘omics’ technologies are gaining traction as enablers of personalized medicine advance at a detailed molecular level, and as such could aid at combining data-driven inductive and symptom-based deductive approaches to accurately represent clinical fibrosis course. An exemplar of heterogeneous fibrosing diseases that can benefit from multivariate data analysis of high-dimensional multiset omics data, and the generation of valid and predictive models for insightful interpretation, is systemic sclerosis (scleroderma, SSc). SSc is a rare chronic disease, of still unknown cause, characterized by multi-organ diffuse fibrosis and vascular abnormalities.

During the SSc fibrotic process, a complex combination of cytokines, chemokines, growth factors, proteases, and extracellular matrix (ECM) constituents are secreted by dermal and resident epithelial cells, all of which add to the inflammatory infiltrate. The linkage of uncontrolled accumulation of ECM, a hallmark of fibrosis, with alterations in inflammatory mediators is concordant with a growing number of studies [6–8]. We and others have shown that a specific chemokines-driven multiscale signaling network (1) promotes attraction of inflammatory cells, (2) directs actions on various target cell types, (3) regulates angiogenesis, and (4) orchestrates tissue remodeling. This polyfunctional heterogeneity of secretions of chemokines and their receptors [9–12] is further evidenced by a number of studies linking the deregulation of chemokine receptor-specific levels to distinct organ and tissue fibrotic cues [11, 13–22].

Clinically, SSc is divided into two subtypes: a more progressive diffuse (dSSc) form and a limited (lSSc) form, depending on the extent of skin fibrosis. This heterogeneity has constrained current treatments that modestly benefit only a subset of patients and hindered predictive analytics of clinical outcomes [23].

The current “gold standard” for assessing severity of SSc in skin is a physical diagnostic test, the modified Rodnan skin score (mRSS). Biologically, the levels of chemokines and their receptors are often elevated in the serum of SSc patients, and fibroblasts (the master regulators of ECM production) from patients show altered chemokine signaling [24–27]. Thus, it is plausible that the variation in gene signatures coding for the extracellular matrix and inflammatory pathways is a reflection of the inherent biology of a given fibrosing disease, representing the pace of SSc instructive cues and hence clinical disease course as captured by mRSS skin score for diagnosis of disease severity.

Using several publicly available datasets, we have applied a novel method, the unsupervised efficiency analysis (UEA), to couple gene signatures to disease pathology and severity based on the stratification of patient-specific indicators of disease progression and outcome. The UEA compares differences in the percentage of overlapping of genes between two disease subsets. Datasets were first analyzed using caGEDA tool [28], which measures microarrays differential gene expression. Then we used the resulting differentially expressed genes to predict disease severity or clinical subtype using a Naïve Bayes classifier and to investigate their associated pathways. Further molecular stratification was used to develop score indices from genes known to be associated with SSc, chronic inflammation, fibrosis, and related canonical pathways. This study provides a principled framework for causal effects estimation from complex high-dimensional data using model informed by inflammation and extracellular matrix gene

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index related to organ and tissue-specific fibrotic cues. Using known key immuno-modulatory and extracellular matrix genes involved in the progression of SSc we have established a panel of 12-genes that could predict disease state with high accuracy to identify three-way relationships between SSc phenotypes, genes and skin score.

**Results**

The objectives of our present study are to test the correlation between chemokines and ECM genes in the samples from patient skin biopsies and identify the genes coding for chemokine genes that govern fibrosis-related alterations in key ECM genes in these patients. To meet this goal, we used a multi-tiered approach which included both unsupervised identification of differentially expressed genes, as well as a second phase that interrogated genes known to be involved in chemokine signaling and ECM production. In both phases, we compared between healthy and dSSc, and between dSSc and ISSc. We first identified a suitable dataset, consisting of gene profile information of skin biopsies using microarrays, which had been deposited to the publicly accessible National Center for Biotechnology Information GEO Database (Accession No. GSE9285) [29]. The sample population includes healthy patients as well as patients diagnosed with dSSc, ISSc, and morphea, with a wide range of mRSS varying across donor demographics (S1 Table). This dataset captures several elements of patient level heterogeneity, including patients with various combinations of gender, age, and racial background.

**Age and skin score association with disease type**

Qualitative and quantitative analysis of clinical features of patients from which skin biopsies for microarray gene expression analysis revealed an intricate interplay between the age of donors and the disease type, as reflected by (Fig 1A–1C) the preponderance of the dSSc type in the most geriatric donors and (Fig 1D, left panel) the typically high skin scores of the dSSc type. In contrast, the biopsy origin appears to have no bearing on the interplay of skin score and demographics (Fig 1B). However, in this study skin score dependence on gender and race, is less conclusive due to gender and race underrepresentation (Fig 1D, middle and right panels).

Bayesian Network was constructed to build a probability model by combining dataset features used in Milano et al. study features and to establish the likelihood of occurrences by using seemingly unlinked attributes. The model displays the interconnection of SSc disease subtype and other factors, such as skin score, age, race, and the origin of biopsy (Fig 2). Amongst those conditions, race demonstrated the lowest level of interdependency, while age and skin score stood as the highest predictors (Fig 2A and S2 Table) of SSc subtype.

The linear projection model developed by Koren et al. [30] which integrates data coordinates with pairwise similarities and/or differences to create a linear transformation displaying the separation and infrastructure between data clusters. Following Koren et al. methods, visual linear transformation of age dependency on SSc disease subtype, exposes definitive clustering of higher skin scores in older dSSc patients (Fig 2B).

**Genomic profiles of healthy vs. dSSc patients differ in their expression of matrix and growth factor signaling genes while dSSc vs. ISSc have a wide range of functions**

We performed unsupervised analyses to compare the expression profiles from healthy and dSSc patients using a total of 54 biopsy samples. Using the J5 statistical test at a threshold of 7.0, we identified 36 genes that were considered differentially expressed between the groups.
Fig 1. Qualitative and quantitative analysis of demographic and clinical characteristics of donor biopsies from microarray gene expression of patient skin biopsies. Shown are bubble charts reflecting the magnitude of the skin score given the race and age of the donors as function of the (A) disease type and (B) biopsy origin as well as the (C) distribution of donor age and disease type or (D) and skin scores as a function of the disease type, gender and race (A = Asian, AA = African American, H = Hispanic, W = White) respectively. Disease type, biopsy origin, race, and sex are color-coordinated, and the size of the bubbles indicate the magnitude of the skin score.

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(Fig 3A and S1 Fig). Among the differentially expressed genes were several [31] that are supported by the literature including COMP [4], FGL2 [32], WIFI [2]. It was also evident that many matrix-related genes were differentially expressed between these two patient groups. We next tested the 36-gene list as a classifier index in a Naïve Bayes model to evaluate its ability to differentiate between genomic profiles of healthy patients from those with dSSc. Classification based upon expression of these genes was highly accurate, with 90% of samples being correctly categorized by the model, sensitivity of 0.871, and specificity of 1.0. We next compared the gene expression profiles of patients with lSSc to those with dSSc. This analysis used a total of 60 samples and, using a J5 threshold of 6.0, identified 64 genes that were significantly differentially expressed between the groups (Fig 3B and S2 Fig). As with the gene list that differentiated between normal and dSSc patients, we tested whether this 64-gene list could be used to classify patients with the two most common clinical subtypes of SSc: dSSc and lSSc. Classification using this panel of genes was accurate for 89% of samples, with sensitivity of 0.871 and specificity of 0.937. The overall theses analyses represent that there are gene expression patterns separating disease subtypes gene expression pattern of this panel is fundamentally heterogeneous. Although the average J5 score seems to be higher in lSSc vs dSSc as opposed to healthy vs dSSc, the gap in the overall levels of gene expression between dSSc and lSSc is reduced as reflected by the shift of both negative and positive J5 score towards the center in lSSc vs dSSc relative to healthy vs dSSc.
Genomic profiles of healthy and dSSc patients differ in their expression of matrix and growth factor signaling genes

To learn more about the pathways and functional networks associated with these genes differentially expressed between healthy and dSSc patients, we performed pathway and impact analysis on the gene list. The pathways with the highest impact factors calculated by Pathway Express are shown in Table 1. Based on impact analysis score, the top three pathways identified were TGF-β signaling pathway, Wnt signaling pathway, and ECM-receptor interaction.

We then performed pathway and impact analysis on the gene list differentially expressed between lSSc and dSSc patients. The top pathway associated with these differentially expressed genes was PPAR signaling with an associated impact factor of 11.982 and was statistically enriched by genes in our list (Table 2). Differentially expressed genes that were present in this pathway were FABP4, LPL, MMP1, and PLIN.

Next we use subset of genes with positive silhouette scores to expand the insights into the relationship between selected gene sets found to be differentially expressed between ISSc and dSSc patient biopsy samples based on J5 analysis by Enrichment analysis using PANTHER.

This silhouette plot shows measure of how well a feature is clustered within a given cluster and the degree of separation from other clusters. A silhouette analysis of healthy vs. dSSc and ISSc vs. dSSc patients reveals distinct relationships between disease tight and differently expressed genes identified by J5 analysis (Fig 4A). Interestingly the scatterplot contrasting the positive silhouette scores healthy vs dSSc as opposed to ISSc shows a high degree of separation (Fig 4B).
An enrichment analysis using PANTHER (Fig 5) of the collective set of genes with positive silhouette scores from the J5 analysis was used to analyze skin-specific protein-protein interaction. These analysis immune and extracellular matrix response and organization.

**Table 1. Pathways associated with differentially expressed genes between healthy and dSSc patient biopsy samples.**

| Rank | Database Name | Pathway Name | Impact Factor | No. Genes in Pathway | No. Input Genes in Pathway | No. Pathway Genes on Chip | % Pathway Genes in Input | Corrected p-value | Sum (PF) | KEGG Pathway ID |
|------|---------------|--------------|---------------|----------------------|----------------------------|---------------------------|------------------------|------------------|----------|----------------|
| 1    | KEGG          | TGF-β signaling pathway | 9.104         | 87                   | 1                          | 71                        | 1.149                  | 0.112591573     | 6.919863899   | 1:04350        |
| 2    | KEGG          | Wnt signaling pathway   | 6.415         | 152                  | 1                          | 123                       | 0.658                  | 0.18712644      | 4.738727096   | 1:04310        |
| 3    | KEGG          | ECM-receptor interaction | 4.477         | 84                   | 1                          | 72                        | 1.19                   | 0.114085731     | 2.306564066   | 1:04512        |
| 4    | KEGG          | Primary immunodeficiency | 4.446         | 35                   | 1                          | 21                        | 2.857                  | 0.034674686     | 1.084276803   | 1:05340        |
| 5    | KEGG          | Ribosome          | 3.255         | 101                  | 1                          | 71                        | 0.99                   | 0.112591573     | 1.071276617   | 1:03010        |
| 6    | KEGG          | Focal adhesion    | 2.563         | 203                  | 1                          | 166                       | 0.493                  | 0.244127768     | 1.153324958   | 1:04510        |

No. Genes in Pathway: Number of genes annotated for pathway, No. Input Genes in Pathway: Number of genes in input list that occur in pathway, No. Pathway Genes on Chip: Number of genes annotated for pathway for which there are probes on microarray chip, % Pathway Genes in Input: Percentage of genes that are annotated for pathway and included in input set, Corrected p-value: FDR-corrected p-value, Sum (PF): Sum of absolute values of perturbation factors.

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Genes that differentiate lSSc and dSSc patients have a wide range of functions

In contrast, the subset of genes with positive silhouette scores will be employed to gain more insights into the relationship between selected gene sets found to be differentially expressed between lSSc and dSSc patient biopsy samples based on J5 analysis by enrichment analysis using PANTHER [33] (Fig 6) shows a high degree of separation but the selected panel of genes/biomarkers correlates significantly with lipid metabolism.

Mining and selection of genes to create predictive gene index (PDI)

Based on recent literature that shows a link between chemokine signaling and expression of extracellular matrix molecules, we tested our hypothesis that a curated list of immunomodulatory and extracellular matrix genes is sufficient to predict disease severity or clinical subtype. We combined pathway- and literature-based methods to define our informed predictive gene index (PDI). We first searched for genes that appeared in pathways related to inflammation (8 pathways) and extracellular matrix (4 pathways), as defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database (www.kegg.jp) (Table 3). In addition, significant findings from literature mining led us to include the following genes: TNC, DCN, FNI, COL1A2, TGFβ, CXCR3, and CXCR4. We chose a panel of 60 genes to use as our PDI, which served as the basis for our predictive modeling approach (Table 4).

Table 2. Pathways associated with differentially expressed genes between lSSc and dSSc patient biopsy samples.

| Database Name | Pathway Name                              | Impact Factor | No. Genes in Pathway | No. Input Genes in Pathway | No. Pathway Genes on Chip | % Pathway Genes in Input | Corrected p-value | Sum (PF) | KEGG Pathway ID |
|---------------|-------------------------------------------|---------------|----------------------|----------------------------|---------------------------|------------------------|------------------|----------|-----------------|
| 1             | KEGG                                      | PPAR signaling pathway | 11.982               | 70                        | 4                         | 52                     | 5.714            | 1.67E-05 | 9.85E-01       |
| 2             | KEGG                                      | Axon guidance  | 7.301                | 129                       | 1                         | 96                     | 0.775            | 2.47E-01 | 5.90E+00       |
| 3             | KEGG                                      | MAPK signaling pathway | 4.294               | 272                       | 1                         | 217                    | 0.368            | 4.75E-01 | 3.55E+00       |
| 4             | KEGG                                      | Primary immunodeficiency | 3.710               | 35                        | 1                         | 21                     | 2.857            | 6.02E-02 | 8.99E-01       |
| 5             | KEGG                                      | Homologous recombinant | 3.585               | 28                        | 1                         | 24                     | 3.571            | 6.84E-02 | 9.03E-01       |
| 6             | KEGG                                      | Bladder cancer | 3.265                | 42                        | 1                         | 36                     | 2.381            | 1.01E-01 | 9.72E-01       |
| 7             | KEGG                                      | Ribosome  | 2.905                | 101                       | 1                         | 71                     | 0.99             | 1.89E-01 | 1.24E+00       |
| 8             | KEGG                                      | TGF-β signaling pathway | 2.700               | 87                        | 1                         | 71                     | 1.149            | 1.89E-01 | 1.04E+00       |
| 9             | KEGG                                      | Hematopoietic cell lineage | 2.660               | 87                        | 1                         | 67                     | 1.149            | 1.80E-01 | 9.43E-01       |
| 10            | KEGG                                      | Alzheimer’s disease  | 2.061               | 178                       | 1                         | 135                    | 0.562            | 3.30E-01 | 9.51E-01       |
| 11            | KEGG                                      | Cytokine- cytokin receptor interaction | 1.857               | 263                       | 1                         | 173                    | 0.38             | 4.01E-01 | 9.43E-01       |
| 12            | KEGG                                      | Pathways in cancer | 1.581                | 330                       | 1                         | 264                    | 0.303            | 5.44E-01 | 9.72E-01       |

No. Genes in Pathway: Number of genes annotated for pathway, No. Input Genes in Pathway: Number of genes in input list that occur in pathway, No. Pathway Genes on Chip: Number of genes annotated for pathway for which there are probes on microarray chip, % Pathway Genes in Input: Percentage of genes that are annotated for pathway and included in input set, Corrected p-value: FDR-corrected p-value, Sum (PF): Sum of absolute values of perturbation factors.

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Inflammation and ECM based Naïve Bayes classification algorithm accurately distinguishes between patient gene expression profiles

We next assessed the ability of our 60-gene PDI to distinguish between gene profiles from healthy and dSSc patient samples, based on gene profile data alone. Using a J5 threshold of 1.4, 18 of the genes from our PDI were identified as being differentially expressed between the healthy and dSSc groups. Among the most significant genes were DCN and LUM (Table 5). PACE analysis indicated that the Naïve Bayes model was significant at PACE 0.045 to J5 1.4 (S3 Fig). The model achieved sensitivity of 0.948 and specificity of 1.0. We also assessed whether our model could accurately differentiate between patients with lSSc and dSSc. When comparing between disease subtype, using J5 threshold of 1.4, 23 genes were differentially expressed, with many being related to major histone compatibility complex (MHC) genes (Table 6). For this comparison, the Naïve Bayes model was significant at PACE 0.05 to J5 1.1 (S4 Fig). The model achieved sensitivity of 0.665 and specificity of 0.814. Lastly, to streamline the predictive gene index, we selected the genes that had the best predictive power to differentiate between high or low severity and among disease subsets, resulting in a final 12-gene index-based classifier that could accurately predict patient outcome based on gene expression profiles from patient skin biopsies (Fig 8). The genes comprising the 12-gene index were PDGFRA, BMP8A, IL15, CXCL5, STAT6, F13A1, CACNG3, ITGAL, COL6A2, HLA-DQA1, HLA-DQB1, and HLA-DRB5.
Discussion

Fibrotic diseases, including systemic sclerosis (scleroderma, SSc), remain debilitating, costly, and painful conditions for thousands of patients. Current treatment strategies often fail in segments of the patient population [34]. These failures have largely been attributed to heterogeneity of disease presentation and progression. In addition, current animal models do not capture the full spectrum of gene expression that underlies various subtypes of human disease [35].

In the absence of definitive biomarkers of SSc pathogenesis, mRSS scores may be confounded by the natural history of disease with age, making comparisons across age groups convoluted (Fig 1). Demographic data analysis has revealed age, but not race, gender and skin origin (Fig 2) to be reliable predictors of SSc disease subtype through a Bayesian network and max-min hill climbing (MMHC) structured learning algorithm (Fig 2A) [36]. Linear projection modeling revealed various ages amongst dSSc patients included in this study, but lSSc patients were found to be older with a narrow range in skin scores (Fig 2B). A study of 67 SSc patients by Perez-Bocanegra et al. also found a likelihood of the lSSc subtype in older patients as well as increased occurrence and more rapid onset of cardiac and pulmonary symptoms with age [37]. More investigation into age and SSc subtype may stand as both a promising diagnostic tool and insight into divergent disease subtype development.

Previous studies have used modeling approaches to identify important biomarker genes and classify SSc patients in a more robust manner than with clinical measurements alone [3, 4]. More recently, investigators have focused on panels comprising a handful of biomarkers to predict disease severity based on gene expression profiling [2, 5]. However, there have been no investigations that focused on the correlation between levels of chemokine and inflammation genes, which are known to be perturbed in disease [38, 39], and the expression levels of ECM genes. Therefore, in the present study we sought to identify the inflammation and ECM genes...
that were most important in predicting patient severity or disease subset, using SSc as a prototype of fibrotic disease.

To meet this goal, we used both unsupervised and literature-based methods to identify gene signatures that could distinguish healthy controls from dSSc patients and dSSc patients from

![Silhouette analysis of genes differentially expressed between dSSc and ISSc patient biopsy samples.](https://doi.org/10.1371/journal.pone.0240986.g006)

**Table 3. KEGG pathways used for selection of genes for predictive gene index (PDI).** All pathways are Homo sapien.

| Pathway ID | Pathway Name                        |
|------------|-------------------------------------|
| hsa04064   | NF-kB signaling pathway             |
| hsa05321   | Inflammatory bowel disease (IBD)    |
| hsa05323   | Rheumatoid arthritis                |
| hsa04062   | Chemokine signaling pathway         |
| hsa04668   | TNF signaling pathway               |
| hsa04010   | MAPK signaling pathway              |
| hsa04610   | Complement and coagulation cascades  |
| hsa04066   | HIF-1 signaling pathway             |
| hsa04510   | Focal adhesion                      |
| hsa04350   | TGF-β signaling pathway             |
| hsa04512   | ECM-receptor interaction            |
| hsa05205   | Proteoglycans in cancer             |

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| Gene Symbol | Gene Name | Associated Pathway |
|-------------|-----------|--------------------|
| TNC         | TNC       | Focal adhesion, ECM-receptor interaction |
| DCN         | DCN       | Proteoglycans in cancer, TGF-β signaling pathway |
| FN1         | FN1       | Focal adhesion, Proteoglycans in cancer, ECM-receptor interaction |
| COL1A2      | COL1A2    | Focal adhesion, ECM-receptor interaction |
| TGFB        | TGFB1     | MAPK signaling pathway, TGF-β signaling pathway, Proteoglycans in cancer, Inflammatory bowel disease (IBD), Rheumatoid arthritis |
| CXCR3       | CXCR3     | Chemokine signaling pathway |
| CXCR4       | CXCR4     | Chemokine signaling pathway |
| A2M         | A2M       | Complement and coagulation cascades |
| ACTB        | ACTB      | Focal adhesion, Proteoglycans in cancer |
| ATP6V1B2    | ATP6V1B2  | ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2 |
| BCAR1       | BCAR1     | Chemokine signaling pathway, Focal adhesion |
| BCL3        | BCL3      | TNF signaling pathway, MAPK signaling pathway |
| BMP8A       | BMP8A     | TGF-β signaling pathway |
| CACNA2D1    | CACNA2D1  | Calcium channel, voltage-dependent, alpha 2/ delta subunit 1 |
| CACNG6      | CACNG6    | Calcium channel, voltage-dependent, gamma subunit 6 |
| CAV2        | CAV2      | Caveolin 2 |
| CCL2        | CCL2      | TNF signaling pathway, Rheumatoid arthritis, Chemokine signaling |
| CCL4        | CCL4      | NF-κB signaling, Chemokine signaling pathway |
| CCR5        | CCR5      | Chemokine signaling pathway |
| CD86        | CD86      | Rheumatoid arthritis |
| COL1A2      | COL1A2    | Focal adhesion, ECM-receptor interaction |
| COL6A2      | COL6A2    | Focal adhesion, ECM-receptor interaction |
| COL6A3      | COL6A3    | Focal adhesion, ECM-receptor interaction |
| CREB3L3     | CREB3L3   | cAMP responsive element binding protein 3-like |
| CXCL5       | CXCL5     | Rheumatoid arthritis, Chemokine signaling, TNF signaling pathway |
| DDX58       | DDX58     | DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 |
| EIF4B       | EIF4B     | eukaryotic translation initiation factor 4B |
| F13A1       | F13A1     | Coagulation factor XIII, A1 polypeptide |
| F7          | F7        | Complement and coagulation cascades |
| FGF19       | FGF19     | MAPK signaling pathway, Proteoglycans in cancer |
| FGF5        | FGF5      | MAPK signaling pathway, Proteoglycans in cancer |
| HCLS1       | HCLS1     | Hematopoietic cell-specific Lyn substrate 1 |
| HLA-DMB     | HLA-DMB   | Major histocompatibility complex, class II, DM |
| HLA-DMA     | HLA-DMA   | Inflammatory bowel disease (IBD), Rheumatoid arthritis |
| HLA-DOA     | HLA-DOA   | Inflammatory bowel disease (IBD), alpha |
| HLA-DPA1    | HLA-DPA1  | Inflammatory bowel disease (IBD), Rheumatoid arthritis |
| HLA-DPB1    | HLA-DPB1  | Inflammatory bowel disease (IBD), Rheumatoid arthritis |

(Continued)
ISSc patients. Our unsupervised, J5-based method revealed several genes that were differentially expressed between healthy and dSSc patients (Fig 3, S1 Fig). In several cases, our methodology confirmed associations that had previously been noted. We found Wnt signaling, TGF-β signaling, and ECM associated genes to be upregulated (Table 1), which has been confirmed at the mRNA and miRNA level in SSc fibroblasts [40]. The Wnt/β-catenin signaling pathway is over activated in SSc patients and expression of WIF1, a Wnt pathway antagonist, is decreased in SSc patients [41], likely through a reactive oxygen species-dependent transcriptional repression mechanism [42]. WIF1 has been posed as part of a biomarker panel for the prediction of skin involvement in dSSc [2]. Therefore, we were not surprised to find that our J5 analysis showed WIF1 was differentially expressed between expression profiles of healthy and dSSc

### Table 4. (Continued)

| Gene Symbol | Gene Name | Associated Pathway [31] |
|-------------|-----------|-------------------------|
| HLA-DQA1    | HLA-DQA1 major histocompatibility complex, class II, DQ alpha 1 | [KO:K06752] Inflammatory bowel disease (IBD), [KO:K06752] Rheumatoid arthritis |
| HLA-DQA2    | HLA-DQA2 major histocompatibility complex, class II, DQ alpha 2 | [KO:K06752] Inflammatory bowel disease (IBD), [KO:K06752] Rheumatoid arthritis |
| HLA-DQB1    | HLA-DQB1 major histocompatibility complex, class II, DQ beta 1 | [KO:K06752] Inflammatory bowel disease (IBD), [KO:K06752] Rheumatoid arthritis |
| HLA-DRB5    | HLA-DRB5 major histocompatibility complex, class II, DR beta 5 | [KO:K06752] Inflammatory bowel disease (IBD), [KO:K06752] Rheumatoid arthritis |
| HRAS        | HRAS Harvey rat sarcoma viral oncogene homolog | [KO:K02833] Chemokine signaling pathway, [KO:K02833] MAPK signaling Pathway, [KO:K02833] Focal adhesion, [KO:K02833] Proteoglycans in cancer |
| IKBKG       | IKBKG inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma | [KO:K07210] MAPK signaling pathway, [KO:K07210] NF-κB signaling pathway, [KO:K07210] Chemokine signaling pathway, [KO:K07210] TNF signaling pathway |
| IL15        | IL15 interleukin 15 | [KO:K05433] TNF signaling pathway, [KO:K05433] Rheumatoid arthritis |
| IL23A       | IL23A interleukin 23, alpha subunit p19 | [KO:K05426] Inflammatory bowel disease (IBD), [KO:K05426] Rheumatoid arthritis |
| ITGAL       | ITGAL integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1 | [KO:K05718] Rheumatoid arthritis |
| ITGB1       | ITGB1 integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) | [KO:K05719] ECM-receptor interaction, [KO:K05719] Focal adhesion, [KO:K05719] Proteoglycans in cancer |
| ITGB2       | ITGB2 integrin, beta 2 (complement component 3 receptor 3 and 4 subunit) | [KO:K06464] Rheumatoid arthritis |
| LAMB1       | LAMB1 laminin, beta 1 | [KO:K05636] Focal adhesion, [KO:K05636] ECM-receptor interaction |
| LUM         | LUM lumican | [KO:K08122] Proteoglycans in cancer |
| MSN         | MSN moesin | [KO:K05763] Proteoglycans in cancer |
| PDGFC       | PDGFC platelet derived growth factor C. | [KO:K05450] Focal adhesion |
| PDGFRA      | PDGFRA platelet-derived growth factor receptor, alpha polypeptide | [KO:K04363] [EC:2.7.10.1] MAPK signaling pathway, [KO:K04363] [EC:2.7.10.1] Focal adhesion |
| PLAUR       | PLAUR plasminogen activator, urokinase | [KO:K01348] [EC:3.4.21.73] Proteoglycans in cancer, [KO:K01348] [EC:3.4.21.73] NF-κB signaling pathway, [KO:K01348] [EC:3.4.21.73] Complement and coagulation cascades |
| RAC2        | RAC2 ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2) | [KO:K07860] Focal adhesion, [KO:K07860] Chemokine signaling pathway, [KO:K07860] MAPK signaling pathway |
| SMAD1       | SMAD1 SMAD family member 1 | [KO:K04676] TGF-β signaling pathway |
| SP1         | SP1 Sp1 transcription factor | [KO:K04684] TGF-β signaling pathway |
| STAT6       | STAT6 signal transducer and activator of transcription 6, interleukin-4 induced | [KO:K11225] Inflammatory bowel disease (IBD) |
| TGFB2       | TGFB2 transforming growth factor, beta receptor II (70/80kDa) | [KO:K04388] [EC:2.7.11.30] TGF-β signaling pathway, [KO:K04388] [EC:2.7.11.30] MAPK signaling pathway |
| TIMP1       | TIMP1 TIMP metalloproteinase inhibitor 1 | [KO:K16451] HIF-1 signaling pathway |
| VAV1        | VAV1 vav 1 guanine nucleotide exchange factor | [KO:K05730] Chemokine signaling pathway, [KO:K05730] Focal adhesion |

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Table 5. Genes from predictive gene index that were differentially expressed between healthy control and dSSc patient biopsy samples.

| J5 Rank | Gene ID   | J5 Score |
|---------|-----------|----------|
| 1       | DCN       | 3.552    |
| 2       | LUM       | -2.729   |
| 3       | HLA-DQA1  | 2.198    |
| 4       | ITGAL     | 2.067    |
| 5       | HLA-DQA2  | 1.907    |
| 6       | LAMB1     | -1.814   |
| 7       | CCL4      | -1.766   |
| 8       | COL6A2    | 1.738    |
| 9       | BCL3      | -1.725   |
| 10      | IKBKG     | -1.723   |
| 11      | F13A1     | 1.635    |
| 12      | TIMP1     | -1.621   |
| 13      | PDGFRA    | 1.599    |
| 14      | COL6A3    | -1.496   |
| 15      | VAV1      | -1.487   |
| 16      | DDX58     | -1.467   |
| 17      | HCLS1     | -1.447   |
| 18      | CACNG6    | -1.405   |

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Table 6. Genes from predictive gene index that were differentially expressed between lSSc and dSSc patient biopsy samples.

| J5 Rank | Gene ID     | J5 Score |
|---------|-------------|----------|
| 1       | HLA-DQA1    | 3.11     |
| 2       | F13A1       | 3.014    |
| 3       | HLA-DRB5    | 2.812    |
| 4       | STAT6       | 2.679    |
| 5       | DCN         | 1.94     |
| 6       | HLA-DQA2    | 2.296    |
| 7       | ITGAL       | 2.236    |
| 8       | ATP6V1B2    | 1.729    |
| 9       | BMP8A       | 1.622    |
| 10      | IL23A       | -1.572   |
| 11      | FGF5        | -1.561   |
| 12      | CACNG6      | -1.522   |
| 13      | CREB3L3     | -1.441   |
| 14      | HRAS        | 1.426    |
| 15      | IKBKG       | -1.397   |
| 16      | LUM         | -1.372   |
| 17      | CACNA2D1    | 1.37     |
| 18      | IL15        | 1.345    |
| 19      | HLA-DQB1    | 1.336    |
| 20      | CCL4        | -1.307   |
| 21      | PDGFRA      | 1.245    |
| 22      | HLA-DPB1    | 1.109    |

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Fig 8. Gene expression grid showing expression of genes in 12-gene panel capable of predicting disease features. Color of boxes indicates directionality of expression differences with red indicating high expression and green indicating low expression. Patient samples highlighted in red were all from dSSc patients and were higher severity (mean mRSS 35.6); samples highlighted in blue were all from lSSc patients and were lower severity (mean mRSS 7.73).

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patients. Our analysis also highlighted ECM protein cartilage oligomeric matrix protein (COMP) (Fig 4, S1 Fig), a gene that is overexpressed in skin of SSc patients [43]. Serum concentration of COMP is associated with mortality risk in SSc patients and it is one gene in a four gene biomarker panel proposed by Farina et al. for assessing the severity of dSSc [3, 5]. We also found that expression of fibrinogen-like protein 2 (FGL2), a glycoprotein that is increased in serum of SSc patients [32], was different between healthy and dSSc patients (Fig 4, S1 Fig). Further analysis showed that the genes characterizing healthy or dSSc profiles were ranked as having high impact on pathways that are critical to the pathogenesis of fibrosis, including TGF-β signaling, Wnt signaling, ECM-receptor interaction, and immunodeficiency [44–46]. Along with these genes, our analysis allowed us to identify several genes that warrant further investigation, including genes related to immune response (IGH, ALOX15B), growth factor signaling (PDGFRL), and extracellular matrix adhesion (LMNB1) (Fig 4, S1 Fig).

Limited (lSSc) and diffuse (dSSc) scleroderma are clinically defined subtypes that differ in both clinical presentation and in terms of which organs are most commonly affected by disease. Patients with dSSc have severe skin involvement, which often rapidly spreads across the body and frequently have cardiac and renal involvement and interstitial lung disease [47, 48]. While skin involvement in lSSc patients is usually confined to the hands and face, these patients are more likely to develop pulmonary arterial hypertension than dSSc patients [49]. In the context of gene expression, previous studies have shown subset-level differences in DNA methylation patterns [50], TGF-β signaling [51], and immune response genes [52] between dSSc and lSSc patients, particularly in fibroblastic gene signatures, the cell type primarily responsible for matrix production [53]. Our J5 analysis identified several genes that were differentially expressed between these disease subtypes (Fig 3, S2 Fig). Matrix metalloproteinases (MMPs) are known to play a central role in fibrosis through their ability to break down ECM constituents. Recent studies have also suggested a role for MMP upregulation in sustained inflammation through the immune cells chemoattraction and proliferation [54, 55], particularly in older individuals [56], suggesting a role of MMP’s in the highly interdependent age and skin score correlations revealed through our Bayesian network projections (Fig 2). Along with several other MMPs and their inhibitors, levels of MMP-1 show close association with SSc, and we found that gene expression differed between dSSc and lSSc patient profiles (Fig 6). Serum levels of MMPs are increased in a subset of patients [57], polymorphisms are associated with various clinical features of disease [58], and anti-MMP1 antibodies are elevated in lSSc patients [59]. To our knowledge, this is the first study indicating that transcript levels of MMP1 may differ between lSSc and dSSc patients. Interestingly, an earlier study showed that serum levels of MMP9 were significantly higher in dSSc than lSSc patients [60]. Hence, further investigation may show MMPs to be a diagnostic marker of SSc disease subtype beyond that of SSc at large.

We also found that lipoprotein lipase (LPL) was differentially expressed between disease subtypes (Fig 6, S2 Fig). A 2005 study found that antibodies against LPL were present in about a third of SSc patients and were associated with organ involvement. Interestingly, the authors found no difference in levels of anti-LPL between dSSc and lSSc patients [61]. Based on the evidence presented within the literature [62], our selected panel of genes/biomarkers differentiating lSSc and dSSc patients correlates significantly with lipid metabolism (Fig 7) which could lead to a minimally invasive means for early detection and monitoring of disease [63, 64].

Similar to the analysis of healthy controls and dSSc patients, our comparison of gene profiles between patients with dSSc and lSSc revealed several novel, potential biomarkers that might be of interest for future study. Our pathway analysis showed PPAR signaling (Table 1) as a top pathway associated with genes expressed between disease subsets. Recent work shows...
that levels of PPAR-γ, which can antagonize TGF-β signaling, are low and dysregulated in patients with SSc [65, 66].

Classification models built using these differentially expressed genes were highly accurate in discerning between severity of disease or disease subtype, indicating that our methods identified panels of genes that were highly correlated with clinical features of interest. However, these gene lists were not rooted in known associations with disease that link to mechanisms of inflammation and extracellular matrix production. Instead of relying on a completely non-parametric approach, we aimed to develop a gene signature that would meaningfully relate to what is known about the development of fibrotic diseases. Based on the pathways identified in the first analyses, we used the available literature to hone in on categories central to the pathogenesis of SSc, extracellular matrix production and inflammation, and mined the literature and known pathways to develop our predictive gene index (PDI). We included specific chemokines and receptors that have been tied to fibrotic diseases, including CXCL3, CXCL4, CCL2, and CCR5 and extracellular matrix molecules that are known to relate to disease such as COL1A2 and LUM [67–70]. Together, our study underscores the importance of the 60 genes (and associated pathways) that we chose in differentiating between healthy and disease, and disease subsets. While it is known that modulation of the ECM and inflammation are key to the development of fibrosis, it was unclear which genes were most closely associated with progression of disease or which defined disease subtypes. The subset of our 60 genes that were differentially expressed between groups were highly accurate in discerning between different conditions when applied to a Naïve Bayes model, indicating that the regulation of these inflammatory and ECM genes may be closely tied to disease pathology. Thus, the ability of our model to faithfully predict severity based on these genes highlights their importance in disease pathogenesis and sheds light on this important aspect of SSc research. Our 12-gene panel represents the genes that might be of the highest relevance to distinguishing between disease states (Fig 8), when considered together.

Furthermore, genes from the predictive gene index identified herein may represent those that should be investigated to develop more clinically representative animal models for therapeutic testing. Recent work has highlighted the fact that murine models commonly used to study SSc do not capture the heterogeneity of human disease [35]. Single gene mutations and knockouts are not sufficient to recapitulate the unique, complex nature of SSc, which leads to poor understanding of disease and therapeutic efficacy. We propose that identification of a gene signature associated with SSc can be considered when developing small animal models with multiple mutations.

The utility of this PDI could be increased if it would be used to predict changes in severity. A longitudinal study would inform whether this model could be used as a prognostic indicator. Furthermore, some ISSc patients progress into dSSc with time. This parallels our findings that the overall discrepancies in gene expression level between dSSc and ISSc skin biopsies is reduced as reflected by the shift of both negative and positive J5 score towards the center in ISSc vs dSSc relative to healthy vs dSSc (Fig 3). A longitudinal study could also be used to evaluate whether any of the “incorrect” prediction classifications from our model that distinguishes ISSc patients from dSSc patients would actually be correct over time and provide insight into those mechanisms of disease progression that currently go undetected.

Another extension of this model is to include other clinical features to stratify patients by characteristics such as organ involvement, autoantibody profile, or to evaluate efficacy of treatments. Future research should investigate the biological mechanisms by which these chemokines and receptors function to modulate production and/or turnover of ECM constituents in disease.
Methods

Data retrieval

Whole-genome DNA microarrays were performed on skin biopsies taken from 34 individuals: 27 from distinct SSc subsets, and 6 healthy controls were used. Sixty-one skin biopsies (multiple biopsies per patient in some cases) and 14 technical replicates were analyzed, resulting in a total of 75 microarray hybridizations. All 75 microarray experiments were included. Skin biopsies were taken from the forearm or lower back. All data are publicly available at the National Center for Biotechnology Information GEO database (http://www.ncbi.nlm.nih.gov/geo; Accession Number: GSE9285) and were originally reported by Milano et al. [29].

Efficiency analysis

Median, raw-intensity, expression values were formatted and annotated by the GPCL-Bioinformatic Analysis Core. Methods for normalization and identification of differentially expressed genes were evaluated using the objective function of maximum internal consistency using efficiency analysis (measured as the consistency in finding the method, including normalization, test and threshold, with the most reproducible set of retained genes during split dataset perturbations). The optimal cut off was selected as the maximum peak of internal consistency at overlap (0 < N3 < N max). The optimized methods for the two comparisons were then applied to the entire data set for each comparison using caGEDA [28]. False discovery rate estimation was conducted using a two-step method [71]. Differentially expressed genes were identified by efficiency analysis (EA), which finds the optimal combination of normalization, transformation, and feature selection techniques to find the most internally consistent set of differentially expressed genes, using AutoEA software [72].

Tests for differential expression

Data transformation and normalization were optimized using efficiency analysis among and between groups. In all comparisons, differentially expressed genes were identified using the J5 test, which is a gene-specific ratio that compares the mean difference in expression intensity between two groups that are being compared to the average mean group difference of all genes in the array. The J5 score was calculated by dividing the mean difference between comparative by the average absolute mean difference of all genes in the data set. Its sign indicates the directionality.

\[
J_{5i} = \frac{\bar{A}_i - \bar{B}_i}{\frac{1}{m} \sum_{j=1}^{m} |A_j - B_j|}
\]

This test is especially useful in cases where there are no accurate estimates of variance, when T-tests are likely to produce high false discovery rates. Analyses were performed using the caGEDA software [28].

Computational prediction

A stringent method was used to explore genes that correlate with the mRSS. Various types of cross-validation, and optimized prediction modeling were undertaken; feature selection (identifying differentially expressed genes) was appropriately nested within the cross-validation loop. Multiple splits between training and test sets were used to minimize stochastic performance due to particular splits. Alternative methods for transformation and normalization were explored using the caGEDA software [28]. Specific classes of prediction modeling
algorithms included Naïve Bayes, logistic regression, random forests, and a genetic-algorithm k of m model in which the model is optimized toward a weighted, achieved classification error. Results were validated using Permutation Achieved Classification Error (PACE) analysis [73], a technique which uses permutations of the dataset to assess the statistical significance of each prediction models' achieved classification errors at given levels. PACE performance statistic of the classifier on true data samples and validates the consistent behavior of the classifier on the same data with randomly reassigned class labels. PACE analysis was use to assess significance of classification results we achieved from published data sets.

Summary scores were generated for each patient based on expression of the genes in our 60-gene predictive gene index. The sum of squared differences for the gene panel was used to rank all samples from high to low. Cut points for classifying new samples in groups along the index were derived based on the accuracy of the resulting classification rules and was evaluated using internal cross-validation. The final reduced set of 12 genes was evaluated as an index-based classifier.

**Functional analysis**

Probe identifications and fold-change values for differentially expressed genes were then submitted to Pathway Express (Onto-Tools, Detroit, MI) for impact analysis [74] and further investigation of known genes, molecular networks, biological pathways, and functions. Impact analysis uses a hypergeometric test to identify canonical pathways that are significantly over-represented in the list of differentially expressed genes compared to their expected representativeness, given the complement of genes on the original microarray, using KEGG pathways as a reference [75]. The iPLEX (San Diego, CA) genotype data analysis was conducted to find an association with the outcome using the Fisher exact test. Further analysis of the differentially expressed genes was conducted with open-access online bioinformatics tools (e.g., DAVID, Frederick, MD) [76] and programs licensed by the University of Pittsburgh Health Sciences Library (e.g., GeneSpring, Agilent technologies, Santa Clara, CA) for cross-referencing and data mining purposes. The pathways and networks identified in Ingenuity Pathway Analysis (IPA) (Qiagen) were used to guide interpretation of the potential function of the differentially expressed genes in relation to the biology of the microarray analyses.

All visualizations were made using R (cran.r-project.org) or Python (www.python.org) programming languages.

**Supporting information**

S1 Table. Descriptive statistics of skin donor biopsy score as function of the donor demographics.

(SDOCX)

S2 Table. Bayesian network model conditional probabilities of disease type.

(SDOCX)

S1 Fig. Gene expression grid showing expression of genes identified by J5 analysis as differentially expressed between genomic profiles of healthy controls and dSSc patient biopsy samples. Color of boxes indicates directionality of expression differences with red indicating high expression and green indicating low expression.

(STIF)

S2 Fig. Gene expression grid showing expression of genes identified by J5 analysis as differentially expressed between genomic profiles of dSSc and lSSc patient biopsy samples. Color of boxes indicates directionality of expression differences with red indicating high expression
and green indicating low expression.

S3 Fig. PACE analysis of Naïve Bayes model for classification of genomic profiles from healthy control compared to dSSc patient biopsy samples. The model was significant at PACE 0.045 up to J5 1.4.

S4 Fig. PACE analysis of Naïve Bayes model for classification of genomic profiles from lSSc compared to dSSc patient biopsy samples. The model was significant at PACE 0.05 up to J5 1.1.

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