Skin biopsy analysis of concurrent keloidal morphoea and systemic sclerosis confirms overlapping pathogenic pathways.

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Running title: Keloidal morphoea genomic analysis

Abstract

Objective

Although localised forms of scleroderma (morphoea) have very different clinical features and outcomes from systemic sclerosis the two conditions can occur together in some patients. In this study we have explored skin gene expression in a series of patients with keloidal morphoea, a distinct clinical variant, concurrently with systemic sclerosis.

Methods

We have compared skin gene expression from the keloidal lesions with that from skin elsewhere. We have also examined a series of patients with diffuse or limited cutaneous SSc without morphoea and some healthy control skin biopsies.

Results

Keloidal morphoea has a distinct gene expression signature that is mainly driven by differential expression of fibroblast related genes compared with other cell types. Indeed, the signature reflects a profibrotic pattern seen in diffuse cutaneous SSc but is much more extreme. We propose that keloidal morphoea skin provides a unique insight into the profibrotic population of cells driving dcSSc.

Conclusion

Understanding the biology of keloidal morphoea may give valuable insight into the molecular and cellular pathology of systemic sclerosis. The discrete nature of keloidal lesions raises the possibility of haematogenous spread and we suggest that the driving cells could represent blood derived cells derived from circulating progenitors.

Key words: morphoea, keloid, scleroderma, genomics, systemic sclerosis

INTRODUCTION

The scleroderma spectrum includes both systemic and localised disease. These are characterised by fibrosis or thickening of the skin but in systemic sclerosis (SSc) there is involvement of internal organs and prominent vasculopathy [1]. The pathobiology of SSc includes vasculopathy, inflammation and fibrosis, all of which play a role in the development of each individual disease phenotype in this heterogenous disorder [2,3]. Conversely, localised scleroderma (LS), also termed morphoea, comprises a group of sclerotic disorders confined to skin, subcutaneous tissue and underlying musculoskeletal structures typically without systemic organ involvement [4].

It is notable that some patients with SSc develop concomitant features of morphoea. There is published literature on the prevalence of morphoea in SSc: suggesting it affects between 3.6 and 6.7% of SSc patients [5,6]. The co-occurrence of SSc and LS adds justification for the terminological linking of these disparate conditions and raises the possibility of shared pathobiology, especially relating to the development of skin fibrosis.

Despite potential mechanistic overlaps in the pathophysiology of the two conditions, recent studies suggest some key differences: data from paediatric LS research suggests that around half develop extracutaneous inflammatory or autoimmune manifestations that are usually distinct from SSc [7,8,9, 10]. The inflammation-driven fibrosis model with genetic and immune aetiological influences is now recognised and precipitants including trauma or skin injury or genomic mosaicism [8]. These data have been advanced by examination of cellular and cytokine signatures both in skin and blood in patients with morphoea, suggesting predominance of a TH1/IFNgamma signature [11].

Nodular or keloidal morphoea is a variant of LS that presents as multiple firm nodules or plaques in a linear or arcuate distribution on the upper trunk and proximal extremities, similar in appearance to keloids or hypertrophic scars. It can be disfiguring and challenging to treat. The terms nodular and keloidal morphoea are sometimes used interchangeably. In this paper we use the term keloidal morphoea (KM). Histological assessment of KM lesions always shows evidence of LS: square edges of biopsy; hyalinised dense collagen with reduced adnexal structures and a variable degree of lymphoplasmocytic perivascular/peri-eccrine and peri-neural infiltrate. In some patients there will also be overlapping features of hypertrophic scarring: increased vascularity, cellularity and dermal collagen fibres oriented parallel and perpendicular to the epidermis; or features of keloid scarring with nodular eosinophilic collagen bundles [12].

In this study, we identify a cohort of SSc cases with both SSc and concomitant keloidal morphoea. We hypothesise that KM may give powerful insights into the drivers of skin fibrosis relevant to SSc

and that 'metastatic' circulating fibroblast progenitors drive the extreme fibrotic gene signatures identified in skin and extrapolate this to the widespread fibroblast activation relevant to skin fibrosis in diffuse cutaneous SSc through GSEA. We have explored this by comparing skin biopsy gene signatures identified in KM lesions within SSc to those of other SSc cases or healthy control skin.

METHODS

Study Design and Patient Recruitment

This was a single centre, prospective observational study comprising of four distinct participant cohorts: SSc with concomitant keloidal morphoea, dcSSc, lcSSc and healthy volunteers (HC).

This study received ethical approval from the NHS Research and Ethics Committee (REC number 6398). The work was performed within the strict General Data Protection Regulations (GDPR) compliant framework of UCL. All participants in this study provided informed consent for their participation, and for the use of their clinical data and samples for research purposes.

Patients with SSc were diagnosed in accordance with the 2013 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria [13] and described as diffuse or limited in terms of skin distribution according to LeRoy 1988 [14] Clinical information for each patient was recorded at the time of the skin biopsy. Demographic, clinical characteristics and current immunosuppressive treatment of the study cohort are summarised in **Table 1**.

Skin Biopsy

4mm skin biopsies were obtained from the dorsal surface of forearm of subjects. As a result, this was clinically normal skin in the HCs and lcSSc, whereas in dcSSc, this clinically involved skin (local mRSS not formally documented). For the patients with concomitant keloidal morphoea, a second biopsy was obtained of the keloidal tissue. All biopsies were stored in RNA*later*™ and stored at -80^{o}C.

RNA sequencing

RNA expression analysis was performed on skin samples stored in RNA*later*™. RNA was isolated using the *RNAeasy* mini kit (Qiagen) according to protocol. All RNAseq was run in one batch on the Illumina NextSeq 550 by Cambridge Genomics Services (Cambridge, UK).

Statistical Analysis

Statistical analysis was carried out using the software R (version 4.0).

For the RNAseq results, normalised FPKM (fragments per kilobase of transcript per million) values were obtained using rlog() function within “DESeq2” Rpackage. Differential gene expression was measured with the Bioconductor “limma” software, and cluster analysis was performed using the Rpackages “ggplot2”, “heatmap.plus” and “edgeR”. Significantly differentially expressed genes were selected as median FPKM ≥ 1 AND fold change (FC) ≥ 1.5 or ≤ 0.68 AND adjusted $p \leq 0.05$ (FDR, Benjamini-Hockberg correction). Where more than 2 groups were analysed, ANOVA was performed, and differentially expressed genes selected with median FPKM ≥ 1 AND adjusted $p \leq 0.05$.

DcSSc disease signatures were obtained from GSE95065, E-MEXP-1214, GSE58095, GSE9285, GSE125362 and GSE76886. DcSSc gene signatures were identified by selecting genes with adjusted $p < 0.1$ and FC ≥ ± 1.5 in ≥ 3 comparisons out of 8 (comprised of the 6 listed studies) [15].

RESULTS

Patient Characteristics

The study recruited 12 patients (4 patients with concomitant SSc and keloidal morphoea, 4 patients with dcSSc and 4 patients with lcSSc) and 4 HCs. Of the patients with keloidal morphoea, 3 (75%) had dcSSc (Table 1). 91.7% of the patients were female, and 50% of the HCs. Mean age across the whole cohort was 55 years (IQR = 16 years, 45.5-61.5), and median disease duration was 9 years (IQR = 9.5 years, 7-16.5 years). 33.3% of patients were anti-topoisomerase-1 autoantibody positive (ATA+, anti-Scl-70), 25% were anti-RNA polymerase III autoantibody positive (ARA+). These patients were managed in line with current treatment guidelines, with 66.7% of patients receiving mycophenolate mofetil.

Gene Expression analysis.

In total, 13,360 genes were identified across the skin biopsy using bulk RNA sequencing and the thresholds for determining expression across replicate samples. These were then used for overall cluster analysis to derive unsupervised groupings that highlighted overall similarities and differences between the individual patient and control skin biopsies.

There was clear separation of keloidal morphoea samples based on gene expression compared to all the other skin samples tested (Figure 1A). DcSSc skin and HC skin also completely separated on the PCA plot, with lcSSc overlapping between the two (consistent with our previous work, see [15]). It is notable that greater separation within the subgroup clusters is observed for dcSSc. This may reflect impact of differences in disease duration, local skin severity or concurrent immunosuppression in this disease subset. In total, 4812 genes which were significantly differentially expressed between all the patient cohorts and HCs. Of those, the genes with the greatest fold change in the KM tissue compared to HC skin biopsies included ADAM12, COL11A1, SFRP4, THBS4 and COMP (Table 2) (Figure 1B).

To explore the differences in gene expression between KM tissue and SSc, we carried out differential expression analysis between the paired samples. Significantly differentially expressed genes were those that had a FC of at least 1.5, and corrected p-value of < 0.05. In total 1245 genes met these criteria as being significantly differentially expressed between the forearm skin biopsies from patients and their paired keloidal morphoea lesions (Figure 2A). The volcano plot emphasises that most of these genes were upregulated in the KM tissue compared to the forearm skin (Figure 2B).

To further understand the function of the genes upregulated in the keloidal tissue lesions compared to the SSc skin, we performed gene set enrichment (Figure 3). The most upregulated processes

included extracellular structure organisation, collagen fibril organisation, and extracellular matrix organisation in keloidal morphoea tissue compared to its paired forearm skin. Of note, there was also significant upregulation of genes associated with skeletal muscle morphogens, ossification and bone development.

We compared our findings to previously reported SSc associated gene expression signatures in the skin from publicly available gene expression datasets for whole skin. The aim was to try to understand the composition of cells that made up the differential gene expression seen between the SSc skin and the keloidal morphoea. Using the datasets previously derived [15], 62.4% of genes within the fibroblast gene data set were significantly upregulated in the keloidal morphoea compared to SSc skin (Figure 3, Table 3), and none of these genes were significantly downregulated. This was a markedly higher proportion of the genes comprising that cell-type signature compared to all the other cell types, of which only 9.6% of genes comprising keratinocyte signatures were upregulated in the keloidal morphoea tissue compared to SSc skin, and 14.4% of the cells making up the melanocyte signature.

DISCUSSION

We have used the powerful molecular methodology of high dimensional analysis of skin gene expression to interrogate the biology of morphoea and systemic sclerosis. Our work specifically focuses on keloidal morphoea lesions that occur in patients with systemic sclerosis. In this way gene expression patterns can be compared between morphoea skin and SSc. This addresses the hypothesis that KM may represent an exaggerated form of skin fibrosis and provide powerful insight into the pathobiology of SSc and fibrosis in general [16]. Our findings suggest that keloidal morphoea lesions reflect the profibrotic mechanisms of SSc but that this is most likely to be a consequence of fibroblasts activation and plausibly these activated profibrotic fibroblasts may be derived from a circulating or migratory population of cells such as those previously implicated in localisation for arthritis in experimental models [17].

Published literature suggests that the lesions of keloidal morphoea may represent an aberrant form of inflammatory response in patients who tend to form keloids or in skin sites with high preference for keloid formation [18], but the rarity and heterogeneity of this condition precludes larger studies. Yamamoto et al found, in a small case series, connective tissue growth factor (CTGF; CCN2) expression at the mRNA and protein levels in fibroblasts in the lesional skin of keloidal morphoea in 3 patients with concurrent keloidal morphoea and diffuse SSc, suggesting that CTGF may play an important role in pathogenesis [19].

Our cell signature analysis shows that most upregulated genes are associated with fibroblasts with little evidence for altered immune cell signatures or vasculopathy. This is consistent with the clinical features of this condition although it is not clear that our findings could be generalised to KM occurring sporadically, outside the context of SSc. Further cellular analysis may be fruitful in future studies using single cell transcriptomic approaches. However, it is notable that the bulk RNAseq signatures identified in KM and SSc in this study are reminiscent of those in other reports. Specifically there is up regulation of genes that have been associated with key pathogenic populations of fibroblasts by others working on skin fibroblasts in SSc using scRNAseq [20,21].

It is notable that the KM gene expression signature represents an exaggerated SSc signature based upon other recent studies [15]. It is also notable that some of the genes have been identified as markers of specific fibroblasts subpopulations in recent scRNAseq analysis. Thus, SFRP2 and SFRP4 highlighted in SSc studies and further suggest aberrant Wnt signalling as a potential pathway important in determining or regulating the profibrotic fibroblasts in both SSc and KM [20,21].

Technological advances in mesenchymal stem cell therapeutics and interest in non-haematopoietic stem cells has led to an appreciation of the role of bone marrow-derived fibroblast progenitors in tissue repair and in pathological scarring states including SSc (particularly interstitial lung disease) and keloid. The concept that fibroblasts can be derived from haematopoietic cells or by epithelial- or endothelial-mesenchymal transformation is not new, though these cells were previously described as a sub-population of PBMCs with haematopoietic stem cells markers including CD34, CD45 and Cd11b [22]. Thus, it is plausible that the activated fibroblasts populations that we identify in keloidal morphoea are at least partially derived from circulating bone marrow derived progenitor cells. It is also possible that fibroblasts may directly migrate to specific sites of keloidal morphoea formation either from local cell populations or via haematogenous spread. Support for the latter comes from previous reports of metastatic fibroblasts determining the location and distribution of joint involvement in experimental arthritis [17]. Although these ideas are attractive and may be central to pathogenesis of keloidal morphoea in SSc, our study cannot directly explore this mechanism and at this stage such concepts remain speculative.

Our study has several strengths. First, RNA sequencing is a powerful tool to look at genome wide expression of mRNA as it directly measures the amount in each sample and has a wide dynamic range so that low and high abundance transcripts may be analysed and compared reliably without the need for external or independent technical validation. In addition, we were able to sample well characterised patients with good clinical annotation. Access to our large cohort also allowed identification of several cases with KM and SSc that gives unique power to our study. This allows within subject comparison as well as across patient analysis. Since inter-subject variability is a major confounder in some transcriptomic studies using microarrays or direct sequencing this is a major methodological strength. It permits interpretation of a small number of samples, and this is supported by the very high degrees of statistical significance that we observe in our keloidal morphoea to SSc skin comparison. By focusing on KM we have selected a clinical subgroup especially suitable for testing our hypothesis that a shared mechanism relevant to fibroblast activation may be identified and that fibroblast populations that arise locally or migrated to the KM lesions may share characteristics relevant to more general skin pathology in SSc.

There are also some limitations in our approach. The small numbers of samples mean that our findings may not be generalisable. In addition, other studies show clear sampling variability and location-specific factors in skin biopsies and fibroblasts that could lead to different findings if other skin biopsy sites were used. The variability between samples is particularly seen in the dcSSc biopsies. This could reflect intrinsic patterns of gene expression in affected skin in dcSSc relating to stage or duration of disease. In addition, differences in immunosuppressive drugs or individual

response to these agents may account for some of the variability. Such factors are well recognised to influence gene expression in other reported studies including dcSSc biopsies. Although bulk RNA sequencing is a robust approach it only allows indirect inference of cell specific signatures. The genes involved may be different in terms of cell number or activation state. This could be explored using single cell methodology such as scRNAseq in future work. Finally, gene expression may not correlate with protein levels, and this would need to be further explored although recent work suggests that proteins often correlate with RNAseq data and that local or circulating levels may be measurable in any future studies [23].

In conclusion, we have used a rare subgroup of SSc patients to highlight the potential value of molecular analysis of small numbers of well characterised samples to gain insight into the molecular pathology of SSc. We show that keloidal morphoea lesions in SSc cases may represent an extreme form of localised skin fibrosis driven by fibroblasts activation and perhaps having relevance to pathobiology of SSc more generally. The present study validates this approach and provides a platform and has generated testable hypothesis that can be explored in future work.

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DISCLOSURES

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TABLES

Table 1. Demographics and clinical characteristics subjects included in the study.

Table 2. The most upregulated genes in keloidal morphoea tissue compared to HC.

Table 3. Enrichment of cell type specific gene signatures in KM samples compared with paired SSc biopsy.

FIGURES AND LEGENDS

Figure 1. Cluster analysis of skin biopsies shows distinct and overlapping patterns of gene expression.

A. Cluster analysis using PCA shows that the paired keloidal morphoea samples clearly separate from the SSc biopsies and that there is overlapping gene expression for limited and diffuse SSc and Healthy controls.

B. Unsupervised cluster analysis of patient level expression of genes that significantly separate KM and other samples reveals groupings that show HC and KM entirely differentiated and with greatest separation. SSc samples largely segregate by subset, with some overlap and are separated from KM and HC.

Figure 2. Differential gene expression between KM and SSc paired skin biopsies.

A. Unsupervised clustering of significantly differentially expressed genes between paired KM and SSc skin biopsies for patients with both diagnoses (n=4) shows complete separation of skin biopsy based upon diagnosis.

B. Volcano plot showing differential gene expression including significantly upregulated or downregulated transcripts. The majority of differentially expressed genes are upregulated in the KM samples compared with SSc skin.

Figure 3. Functional genomic analysis of gene sets enriched in keloidal morphoea (KM) skin biopsies.

Overlapping sets of differentially expressed genes using cell type specific signatures are shown for fibroblasts genes showing that most upregulated genes are within the fibroblasts gene set (A) and

only a small proportion of keratinocyte associated genes are upregulated in KM (B). Pathway analysis enrichment scores and significance are shown in panel C. As expected, extracellular matrix pathways have the highest enrichment score providing validation of the likely central role of the differentially expressed genes in the fibrotic pathology of both KM and SSc.

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Table 1. Demographics and clinical characteristics subjects included in the study.

	SSc + keloid	DcSSc	LcSSc	HC
Age (yrs)	49.5 (14.5)	45.5 (19)	55.5 (15)	63.5 (16.5)
DcSSc (%)	3 (75%)			
Gender (%F)	4 (100%)	4 (100%)	3 (75%)	2 (50%)
Disease duration (yrs)	7.5 (4.5)	7.5 (6.5)	22 (14)	
mRSS	11.5 (8)	16 (5)	6 (3)	
Autoantibody				
ATA	3	1		
ARA	1	2		
ACA			2	
Other		1	2	
Immunosuppression				
MMF	4	4		
MTX	1	1		
Rituximab	1			
Tocilizumab	1			
Organ involvement				
ILD	2	2	1	
PAH				
Cardiac		1		
Inflammatory arthritis	1	1		
Digital ulcers	2	2	2	

Median age and disease duration and skin score (mRSS) reported, IQR in brackets. Abbreviations: Anti-topoisomerase 1 antibody (ATA), Anti RNA polymerase III antibody (ARA), Anticentromere antibody (ACA), Mycophenolate mofetil (MMF), Methotrexate (MTX), Interstitial lung disease (ILD), Pulmonary arterial hypertension (PAH).

Table 2. The most upregulated genes in keloidal morphoea tissue compared to HC.

	Fold change compared to HC		
	lcSSc vs HC	dcSSc vs HC	Keloid vs HC
ADAM12	1.288	1.710	73.343
COL11A1	2.301	1.944	26.769
CILP2	1.054	1.150	23.255
ACAN	1.404	1.693	22.913
CPXM1	1.446	2.026	22.057
ASPN	0.964	1.205	19.203
SFRP4	1.182	2.224	17.507
THBS4	0.944	1.875	15.850
COMP	1.259	1.891	13.799
COL1A1	0.889	1.480	11.224
COL8A1	1.054	1.615	10.695
COL5A2	1.014	1.289	10.328
COL3A1	0.874	1.213	9.966
OGN	0.644	0.801	8.628
COL10A1	1.093	1.146	8.362
COL1A2	0.800	1.147	7.651
FBN2	1.128	1.190	7.419
GALNT5	0.866	0.956	7.260
TENM3	0.997	1.076	7.125
LRRC15	1.143	1.356	7.071
MDK	1.064	1.094	6.932
LAMP5	1.113	1.307	6.814
SULF1	1.029	1.471	6.672
SPARC	0.844	1.160	6.648
P4HA3	1.003	1.223	6.277

COL6A3	1.030	1.304	6.225
TGFB3	0.966	1.187	6.212
COL12A1	0.769	0.919	6.069
COL5A1	0.852	1.256	5.986
ADAMTS2	0.831	1.238	5.980

Fold change for dcSSc and lcSSc against HC also shown.

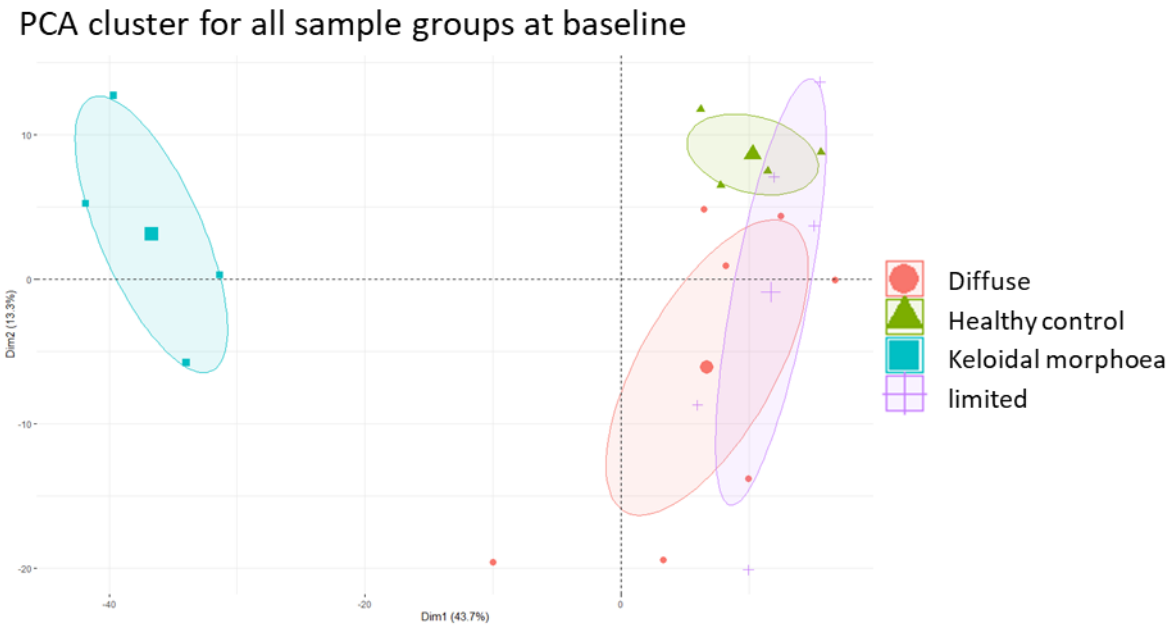
Table 3 Enrichment of cell type specific gene signatures in KM samples compared with paired SSc biopsy.

	Percentage of signature upregulated in keloidal morphoea tissue compared to paired sample		Percentage of signature downregulated in keloidal morphoea tissue compared to paired sample	
	Number genes	%	Number genes	%
B cells	0	0%	0	0%
immature DCs	5	4%	3	2.4%
M1 cells	7	5.6%	0	0
M2 cells	6	4.8%	1	0.8%
mature DCs	5	4%	3	2.4%
melanocytes	18	14.4%	2	1.6%
monocytes	5	4%	1	0.8%
T cells	3	2.4%	6	4.8%
fibroblasts	**78**	**62.4%**	**0**	**0%**
keratinocytes	12	9.6%	20	16%

Number and percentage of genes in cell type signature upregulated and downregulated in keloidal morphoea when compared to paired SSc skin.

Figure 1

A


PCA cluster for all sample groups at baseline
Dim2 (13.3%)
Dim1 (43.7%)
Diffuse
Healthy control
Keloidal morphoea
limited

B

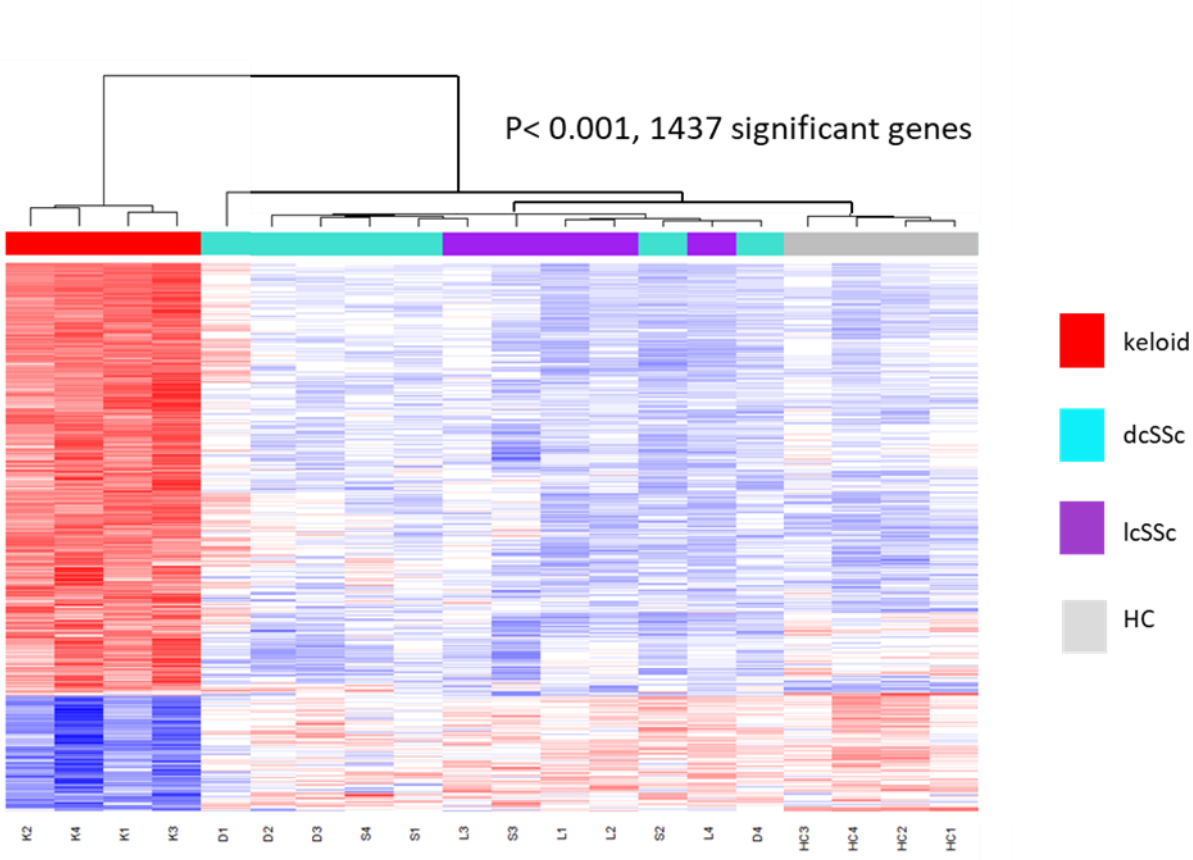
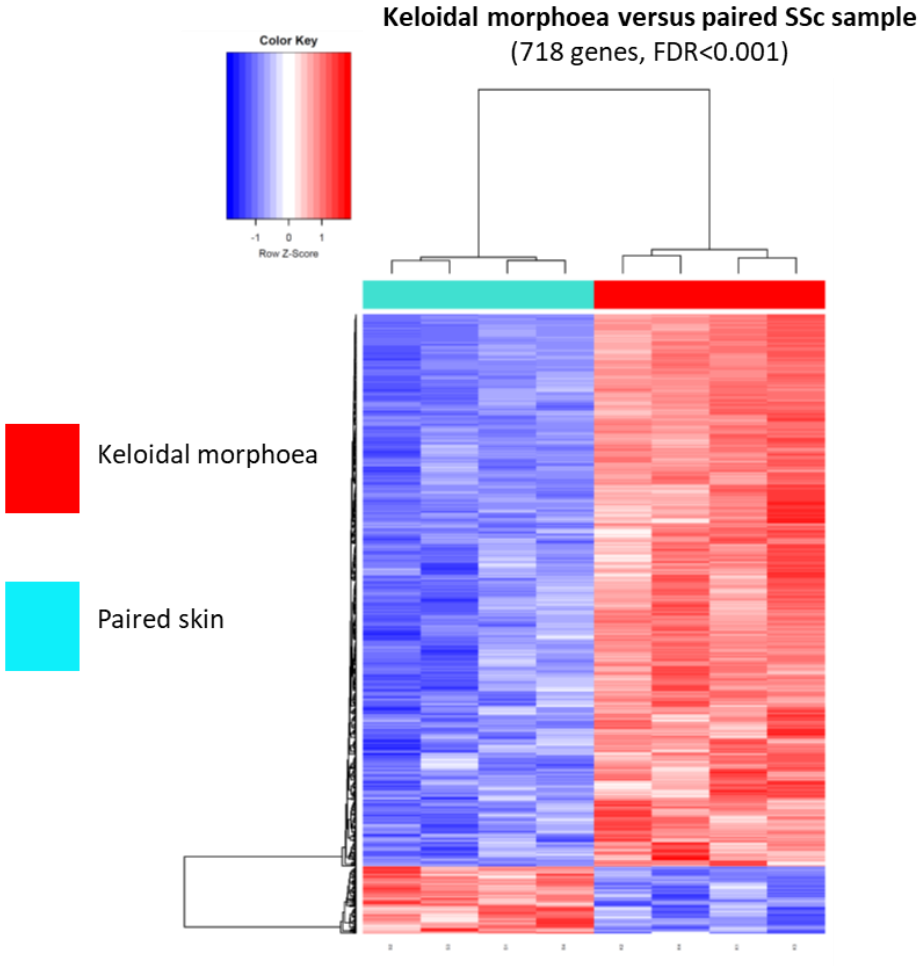

P< 0.001, 1437 significant genes
K2
K4
K1
K3
D1
D2
D3
S4
S1
L3
S3
L1
L2
S2
L4
D4
HC3
HC4
HC2
HC1
keloid
dcSSc
lcSSc
HC

Figure 2

A


Keloidal morphoea versus paired SSc sample
(718 genes, FDR<0.001)
Color Key
-1
0
1
Row Z-Score
Keloidal morphoea
Paired skin

B

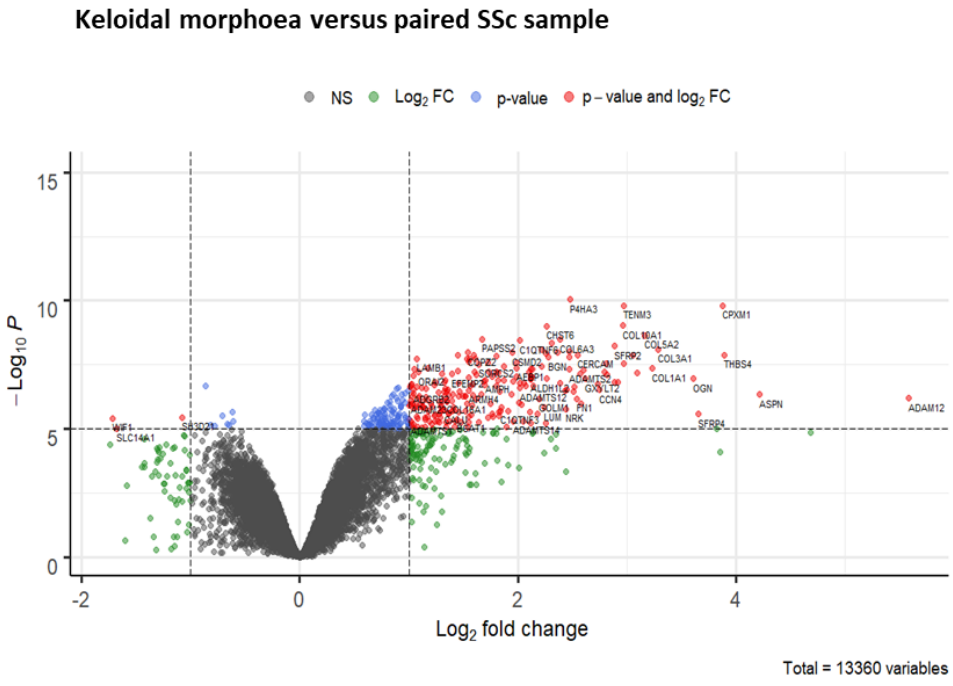
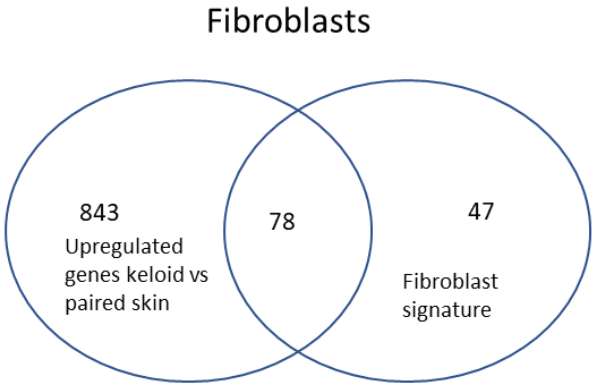
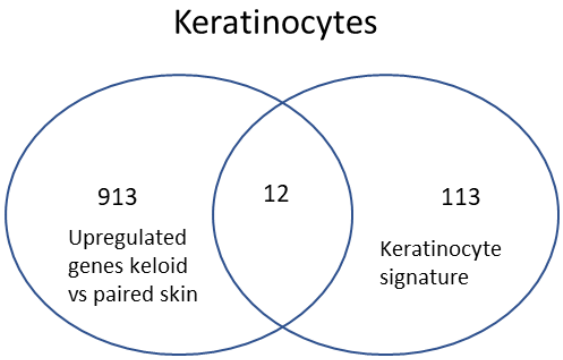

Keloidal morphoea versus paired SSc sample
NS
Log2 FC
p-value
p – value and log2 FC
15
10
5
0
−Log10 P
-2
0
2
4
Log2 fold change
Total = 13360 variables

Figure 3

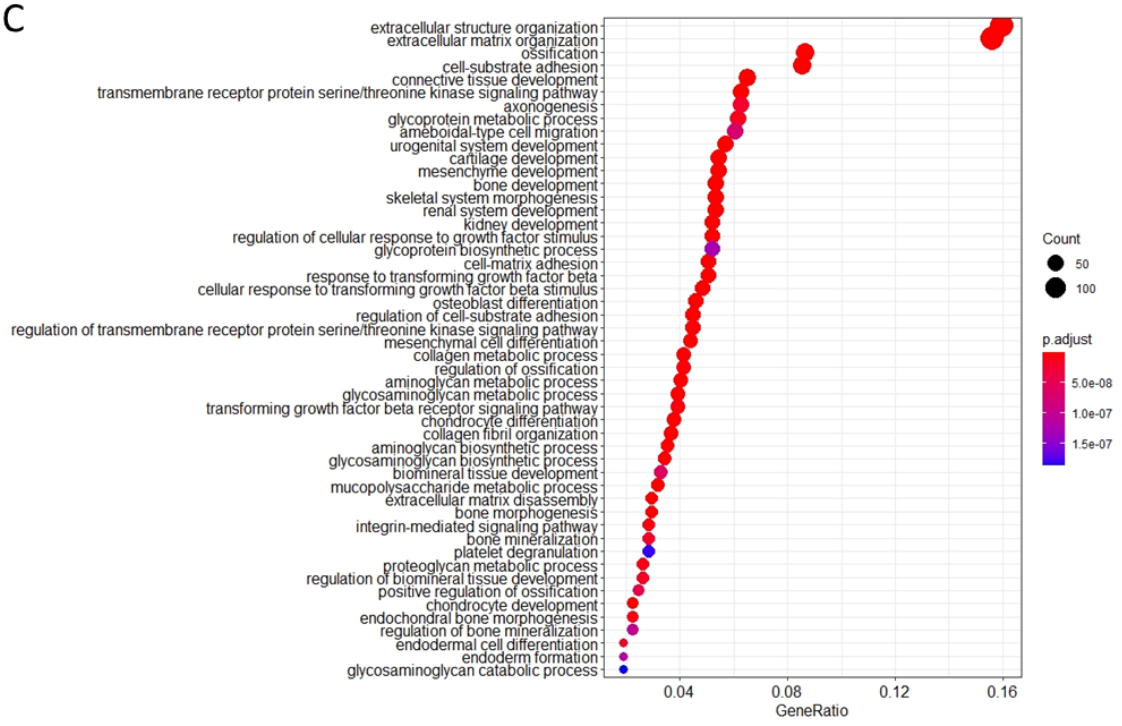
A


Fibroblasts
843
Upregulated genes keloid vs paired skin
78
47
Fibroblast signature

B


Keratinocytes
913
Upregulated genes keloid vs paired skin
12
113
Keratinocyte signature

C


extracellular structure organization
extracellular matrix organization
ossification
cell-substrate adhesion
connective tissue development
transmembrane receptor protein serine/threonine kinase signaling pathway
axonogenesis
glycoprotein metabolic process
ameboidal-type cell migration
urogenital system development
cartilage development
mesenchyme development
bone development
skeletal system morphogenesis
renal system development
kidney development
regulation of cellular response to growth factor stimulus
glycoprotein biosynthetic process
cell-matrix adhesion
response to transforming growth factor beta
cellular response to transforming growth factor beta stimulus
osteoblast differentiation
regulation of cell-substrate adhesion
regulation of transmembrane receptor protein serine/threonine kinase signaling pathway
mesenchymal cell differentiation
collagen metabolic process
regulation of ossification
aminoglycan metabolic process
glycosaminoglycan metabolic process
transforming growth factor beta receptor signaling pathway
chondrocyte differentiation
collagen fibril organization
aminoglycan biosynthetic process
glycosaminoglycan biosynthetic process
biomineral tissue development
mucopolysaccharide metabolic process
extracellular matrix disassembly
bone morphogenesis
integrin-mediated signaling pathway
bone mineralization
platelet degranulation
proteoglycan metabolic process
regulation of biomineral tissue development
positive regulation of ossification
chondrocyte development
endochondral bone morphogenesis
regulation of bone mineralization
endodermal cell differentiation
endoderm formation
glycosaminoglycan catabolic process
0.04
0.08
0.12
0.16
GeneRatio
Count
50
100
p.adjust
5.0e-08
1.0e-07
1.5e-07