Clinical Rheumatology (2019) 38:933–938
https://doi.org/10.1007/s10067-018-4298-5

BRIEF REPORT

Dysregulation of the Wnt signaling pathway in South African patients with diffuse systemic sclerosis

Jacqueline Frost[1,2] · Xavier Estivill[3,4,5] · Michèle Ramsay[1,6] · Mohammed Tikly[7]

Received: 14 May 2018 / Revised: 11 September 2018 / Accepted: 13 September 2018 / Published online: 20 September 2018

Abstract

The objective was to explore changes in gene expression in Wnt pathway genes in skin samples of black South Africans with diffuse cutaneous systemic sclerosis (dcSSc). Affected (forearm) and unaffected (upper back) skin samples of eight Black South Africans with active early dcSSc were compared to skin samples from seven ethnically matched control subjects. The Wnt Pathway Plus RT^2 Profiler qPCR Array was used to determine gene expression and analyzed for differential expression between cases and controls. Selective validation was done using single-gene TaqMan assays. Several genes were similarly upregulated in both affected and unaffected skin of the dcSSc patients compared to controls. These included the Wnt ligands *WNT7A* and *WNT10A*, the frizzled receptors *FZD8* and *FZD9*, intracellular signaling proteins *AXIN1* and *AXIN2,* and the pathway target genes *FGF4* and *MMP7*. Principal component analysis revealed patients clustering into two groups, which co-segregated with clinical features of interstitial lung disease and/or inflammatory myopathy, or the absence of an inflammation phenotype. These two groups showed paradoxical gene expression of the genes *TCF7*, *SOX17*, and *FRZB* in affected and unaffected skin. This study provides further evidence of dysregulation of gene expression at various levels of the Wnt signaling pathway in dcSSc. Moreover, principal component analysis showed two distinct patient clusters of gene expression, which co-segregated with the presence or absence of clinical inflammatory features, and may reflect different pathological pathways in dcSSc.

Keywords African · Fibrosis · Gene expression · Systemic sclerosis · Wnt pathway

The fibroblast and its activated phenotype, the myofibroblast, play a pivotal role in the overproduction of extracellular matrix (ECM) and fibrosis. Fibrosis results from increased synthesis of collagen and other ECM proteins, coupled with reduced degradation of the ECM in systemic sclerosis (SSc) [1]. Much of the present evidence points to the transforming growth factor-beta (TGF-β)-SMAD signaling pathway playing a central role in myofibroblast activity and fibrosis in SSc.

The Wnt signaling pathway, one of the morphogen pathways, is primarily involved in embryogenesis. It is also known to modulate fibroblast function directly and indirectly via crosstalk between the canonical Wnt/β-catenin and TGF-β

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10067-018-4298-5) contains supplementary material, which is available to authorized users.

✉ Jacqueline Frost
Jacqueline.frost@mssm.edu

[1] Division of Human Genetics, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg 2001, South Africa

[2] Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, 1425 Madison Avenue, New York City, NY 10029, USA

[3] Genomics and Disease Group, Bioinformatics and Genomics Program, Centre for Genomic Regulation, The Barcelona Institute of Science and Technology, 08003 Barcelona, Spain

[4] Pompeu Fabra University, 08003 Barcelona, Spain

[5] Experimental Genetics Division, Sidra Medical and Research Center, Doha 26999, Qatar

[6] University of the Witwatersrand, Sydney Brenner Institute for Molecular Bioscience, Johannesburg 2001, South Africa

[7] Division of Rheumatology, School of Clinical Medicine, University of the Witwatersrand, Johannesburg 2001, South Africa

signaling pathways [2]. Dysregulation of the canonical Wnt pathway has been observed in various fibrotic disorders, including SSc [3].

Dysregulation of the canonical Wnt pathway has been observed in both animal models and human studies in SSc. In the *tsk* mouse model of SSc, perturbations in Wnt/β-catenin signaling have been shown to aggravate markers of injury and fibrosis in a variety of different tissues [4]. Increased *Wnt10b* expression has been observed in mice with bleomycin-induced fibrosis [5]. Variable dysregulation of the canonical Wnt pathway has been observed in human skin samples of SSc, such as dysregulation of *WIF1* in a study of 9 patients [6]. In a further study using microarray technology, of the 1800 differentially expressed genes, 4 were of the Wnt pathway including *WIF1*, *FRZB*, *FZD7*, and *SFRP4* [7]. Moreover, TGF-β was found to stimulate the canonical Wnt pathway by decreasing expression of the Wnt antagonist *DKK1* [8].

To better understand the Wnt signaling changes in diffuse cutaneous SSc (dcSSc), and in the absence of studies in black Africans in whom dcSSc is the predominant subset, we examined differential gene expression of 84 genes in the Wnt signaling pathway in black South Africans with dcSSc. The study was approved by the Human Research Ethics Committee (Medical), Faculty of Health Sciences, University of the Witwatersrand, and all participants consented in accordance with the Declaration of Helsinki guidelines.

Materials and methods

Nine black South African patients who fulfilled the 1980 ACR SSc classification criteria [9], had dcSSc as defined by LeRoy and Medsger [10], and ≤ 5-year disease duration were recruited for the study. Each patient underwent two 4-mm punch biopsies from (1) clinically affected skin (henceforth referred to as "affected skin"), 4 cm proximal to the ulna styloid on the dorsum of the left forearm, and (2) clinically unaffected skin (henceforth referred to as "unaffected skin") from the upper back. Control skin samples were obtained from seven otherwise healthy individuals undergoing plastic surgery, three from the breast and four from the forearm regions. All skin samples were stored in RNAlater (Qiagen) then transferred into Qiazol (Qiagen) before RNA was purified using the RNeasy miRNA kit (Qiagen). RNA quality was assessed using the Bioanalyzer (Agilent Technologies). Samples were labeled as "P" for affected skin and "B" for unaffected skin samples from patients and "C" for control skin samples.

Reverse transcription and Wnt qPCR Array

RT^2 PreAMP cDNA Synthesis Kit and RT^2 PreAMP Pathway primer mix specific for the Wnt pathway (Qiagen) were used to amplify cDNA after which samples were added to a Human Wnt Signaling Pathway RT^2 Profiler qPCR Array (Qiagen) and run on the ABI 7900HT Real-Time PCR machine (Applied Biosystems).

Fig. 1 Principal component analysis plots and heatmap showing ▶ significantly differentially expressed Wnt pathway genes in SSc patients compared to controls. **a** This plot clearly shows the two sub-groups of SSc patient samples clustering separately from each other and from the control samples. **b** Affected SSc skin (forearm) samples (P1–P9) and the controls, with two clear clusters forming for the patients. The P1 sample clusters with the control samples. **c** Unaffected SSc skin (back) samples (1B–9B) and the controls, once again with two distinct clusters forming for the patient samples. The B1 sample does not cluster with either patient cluster or the control cluster. As the two samples from patient 1 do not cluster in the two patient clusters, data from this patient were excluded from further analyses. **d** The colored bars represent the gene clusters observed where the significantly downregulated genes in SSc patients (affected and unaffected skin) compared to controls are in the green cluster. Genes that are significantly upregulated in SSc patients (affected and unaffected skin) compared to controls are in the red cluster. Heterogeneous upregulation of genes in SSc skin is observed in the blue gene cluster. The orange and purple gene clusters are those genes differentially expressed in a pattern that suggests these genes could be the cause of differentiating the patient into the distinct clusters as observed in the PCA plots

Validation

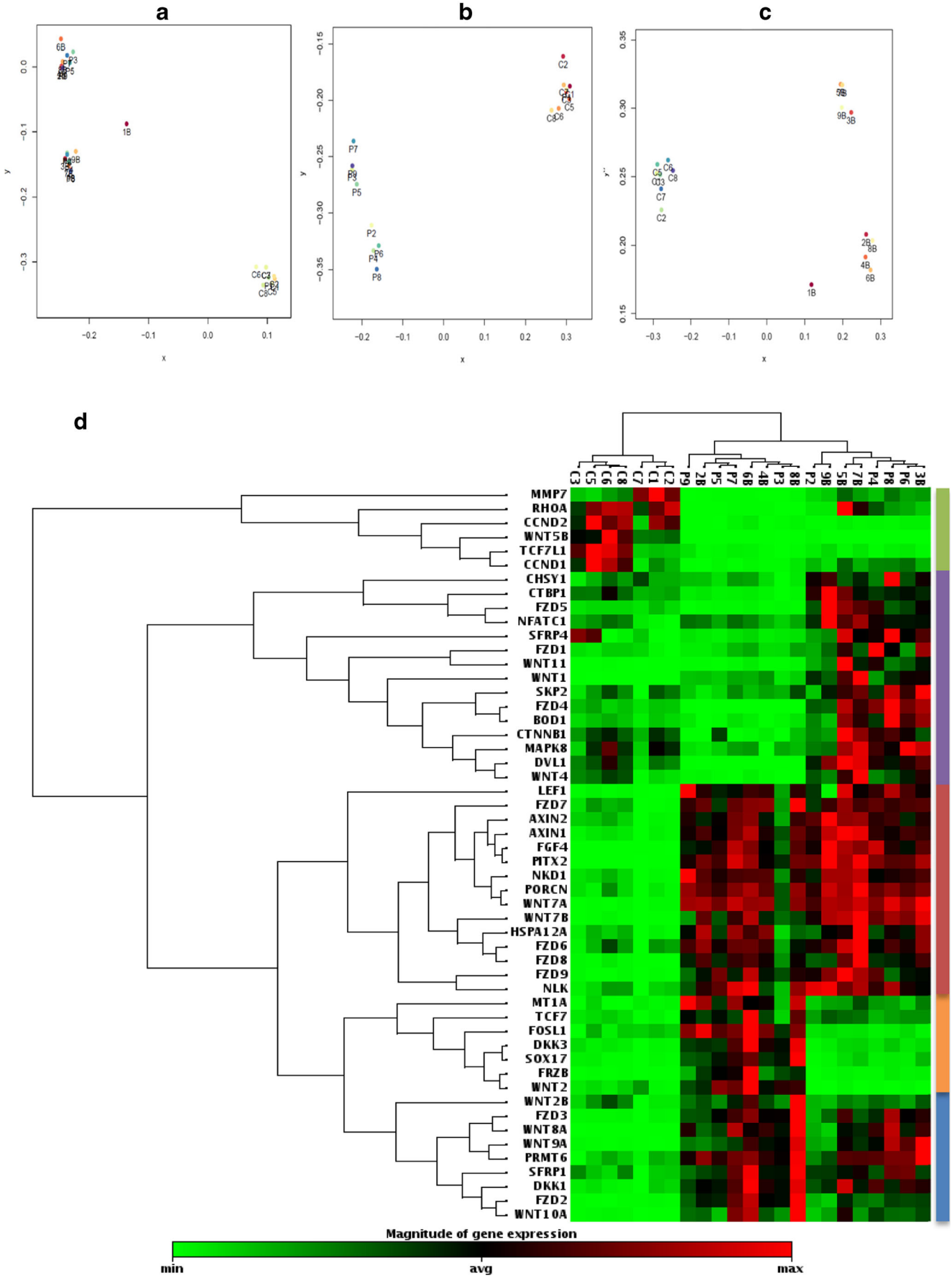
Reverse transcription was done using the High Capacity RNA-to-cDNA kit (Thermo-Fisher Scientific). Pre-designed TaqMan gene expression assays were obtained from Thermo-Fisher Scientific. Samples were run in triplicate for each assay on the ABI 7900HT Real-Time PCR machine. Genes were selected based on the magnitude of differential expression (fold change) from the qPCR array results, statistical significance (p value), and their purported role in the Wnt signaling pathway.

Data analysis

Wnt qPCR array data were analyzed using the R/Bioconductor package HTqPCR. Gene expression was normalized to the expression of two housekeeping genes (*ACTB* and *HPRT1*); thereafter, differential expression calculated using the $2^{-\Delta\Delta Ct}$ method was considered significant with a fold change > 2 and an adjusted p value ≤ 0.05 (Benjamini–Hochberg rule). TaqMan assay raw data were analyzed using relative quantification software on the ABI 7900HT Real-Time PCR machine, followed by differential expression analysis using the R/Bioconductor software packages, ReadqPCR and NormqPCR.

Results

On the basis of the findings on principal component analysis (PCA), patient 1 was excluded because the affected skin (P1)


a
b
c
d
MMP7
RHOA
CCND2
WNT5B
TCF7L1
CCND1
CHSY1
CTBP1
FZD5
NFATC1
SFRP4
FZD1
WNT11
WNT1
SKP2
FZD4
BOD1
CTNNB1
MAPK8
DVL1
WNT4
LEF1
FZD7
AXIN2
AXIN1
FGF4
PITX2
NKD1
PORCN
WNT7A
WNT7B
HSPA12A
FZD6
FZD8
FZD9
NLK
MT1A
TCF7
FOSL1
DKK3
SOX17
FRZB
WNT2
WNT2B
FZD3
WNT8A
WNT9A
PRMT6
SFRP1
DKK1
FZD2
WNT10A
Magnitude of gene expression
min
avg
max

was positioned within the control samples (C) and the unaffected skin sample (1B) did not cluster with any group (Fig.1a). The remaining eight patients included in the comparative analysis were mainly female ($n = 7$), with a mean (SD) disease duration of 31.5 months (15.6), modified Rodnan skin score of 18.3 (11.7), and active disease as defined by the Valentini disease activity score [11] of 4.2 (1.7). Other clinical features of note were interstitial lung disease (ILD) in four patients and inflammatory myopathy (IM) in three cases. All had been treated with immunosuppressive agents. Six patients had been treated for either ILD or severe skin disease with a combination of low-dose oral prednisone (10 mg/day) and intravenous cyclophosphamide for 6 months, followed by either azathioprine in four cases or mycophenolate mofetil in two cases. Two patients had been treated with oral methotrexate. Details of patient demographic data can be found in Supplementary Table 1.

Further PCA analysis showed from the eight patients, clustering of gene expression into two distinct groups, each containing both affected and unaffected skin samples (Fig. 1a). Affected skin samples P2, P4, P6, and P8 (group 1) clustered separately from the second cluster P3, P5, P7, and P9 (group 2) (Fig. 1b) and the unaffected skin samples for the same patients formed two clusters (Fig. 1c).

Closer examination of the heatmap (Fig.1d) showed several groups of genes with different overall expression patterns, color-coded according to the observed differential expression. Fifteen genes were significantly upregulated in both affected and unaffected skin in dcSSc samples compared to controls (red cluster) (Supplementary Table 2). These included genes of Wnt signaling molecules *WNT7A* and *WNT7B*; frizzled receptors *FZD6, FZD8*, and *FZD9*; and Wnt pathway antagonists *AXIN1* and *AXIN2*. The genes that showed the largest magnitude of increased expression were *FGF4* and *PITX2*. Conversely, *WNT5B*, a known initiator of the non-canonical Wnt pathway, and *CCND1*, *CCND2*, and *MMP7*, all of which are downstream target genes of the activated Wnt signaling pathway, were downregulated (green cluster) in both affected and unaffected skin (Supplementary Table 2). The genes that were validated using the TaqMan assay were *WNT7A*, *WNT5B*, *FZD2, AXIN1*, and *LEF1*. In all cases, the direction of the differential expression was consistent with the original qPCR array findings, although the magnitude (fold change) was lower with the TaqMan assay (Table 1).

The pattern of gene expression observed in the orange and purple gene clusters (Fig. 1d) show distinct differences for affected and unaffected skin samples according to patient groups 1 and 2. There was paradoxical dysregulation of the genes *TCF7*, *SOX17*, and *FRZB* for the two patient clusters, groups 1 and 2. Of interest is that the gene expression clustering in patient group 1 co-segregated with patients who had either inflammatory features of interstitial lung disease (ILD) and/or inflammatory myopathy (IM), or the absence thereof (group 2).

Within the control group, there was variable gene expression, including *MYC, WIF1, EP300*, and *VANGL2*. These differences appear to be related to the site of the skin sample, the forearm (samples 1, 2, 6, and 7) and breast (samples 3, 5, and 8) (Fig. 1d).

Discussion

The present study in black South Africans with early active dcSSc provides further evidence of dysregulation of the canonical Wnt pathway in SSc. For 18 of the 84 genes, there was no significant difference in gene expression between affected and unaffected skin, as previously reported [6].

Of the Wnt ligands that were upregulated, *WNT7A* and *WNT10A* were upregulated by 8- and 6-fold, respectively in both affected and unaffected skin. Activation of *WNT7A* and *WNT7B* has previously been shown to be profibrotic by increasing the expression of *COL1* and therefore increasing collagen synthesis in multipotent mural mesenchymal progenitor cells [12]. WNT10A is an inducer of both the canonical and the non-canonical Wnt pathway. Although *WNT10A* has not been previously found to be upregulated in skin biopsy specimens of SSc patients, it has been reported to have a profibrotic role in the development of idiopathic pulmonary fibrosis [13].

Several of the frizzled receptor genes were upregulated in both affected and unaffected skin, most notably *FZD9* and *FZD8*. Frizzled receptor activation is known to block β-catenin degradation, the latter playing a critical role in target gene transcription via TCF/LEF1 binding in the nucleus. *FZD8* expression was shown to be induced in the

Table 1 Differential expression values for the five genes chosen for validation, observed in both the qPCR array experiment and the TaqMan validation experiments

	Affected skin vs controls		Unaffected skin vs controls	
Gene	Relative fold change (qPCR array)	Relative fold change (TaqMan)	Relative fold change (qPCR array)	Relative fold change (TaqMan)
AXIN1	4.24	3.63	4.67	1.90
LEF1	6.09	2.80	5.67	1.19
FZD2	6.00	1.11	6.21	2.64
WNT7A	8.09	1.51	8.30	2.59
WNT5B	− 9.59	− 1.55	− 9.97	− 3.10

bleomycin-induced fibrosis mouse model, and, conversely, lung fibrosis was reduced in *FZD8*-deficient mice [14]. Moreover, several of the frizzled genes have been found to be upregulated in experimental renal fibrosis following obstructive injury [15]. Together, these findings indicate that upregulation of frizzled receptor genes play a profibrotic role.

AXIN1 and *AXIN2* are intracellular negative regulators of Wnt signaling. They function by binding to GSK3β and β-catenin, promoting the degradation of β-catenin [16]. In the present study, although *AXIN1* and *AXIN2* were upregulated in both affected and unaffected skin, their gene expression was numerically higher in unaffected skin suggesting that the upregulation of these AXIN genes is a possible compensatory phenomenon in an attempt to reduce fibrosis.

Several of the downstream target genes showed altered gene expression. Fibroblast growth factor 4 (*FGF4*) displayed the highest magnitude of differential expression, 20-fold higher in affected and unaffected skin compared to controls. The fibroblast growth factors are a family of peptides with pleotropic vascular and connective tissue effects. They play an important role in early embryonic development with respect to cell differentiation, morphogenesis, and proliferation but also contribute to wound healing. *FGF2* has been observed to be upregulated in Asian patients with both dcSSc and limited cutaneous SSc [17].

The downregulation of *MMP7* by 3-fold in the present study is intriguing since most other studies have shown upregulation. In a bleomycin-induced fibrosis model, they confirmed increased MMP7 protein expression by epithelial cells and also noted that $MMP7^{-/-}$ knockouts were protected from bleomycin-induced fibrosis [18].

The clustering of genes that co-segregate with the presence of clinical inflammatory features of ILD/IM or the absence thereof is akin to the previously proposed molecular phenotypes of "diffuse-proliferative" and "inflammatory" gene expression patterns [19]. In the present study, there was paradoxical dysregulation of *TCF7*, *SOX17*, and *FRZB*, wherein one cluster (patient group 1) of these genes is upregulated in affected skin and downregulated in unaffected skin and vice versa for the other cluster (patient group 2). The genes that show these paradoxical expressions have not been previously implicated in fibrosis. In our study, there was both increased and decreased expression of *TCF7*; this gene, when coupled to LEF1, plays an important role as a nuclear transcription factor in the Wnt/β-catenin response. Similarly, *SOX17* was downregulated in affected skin samples for patients 2, 4, 6, and 8. *SOX17* acts as a transcription regulator that binds target promoter DNA and promotes degradation of activated CTNNB1 thereby inhibiting the Wnt signaling pathway [20]. It would be interesting to investigate if the gene signatures observed in this study could be used as biomarkers of response to immunosuppressive therapy. This has been shown with a different set of genes in a prospective study in which the immunosuppressant mycophenolate mofetil was used.

Some of the limitations of the study were that we were unable to do immunohistochemistry to localize and correlate gene and protein expression to specific cells. We used whole skin punch biopsy samples that contain various cell types including fibroblasts, epithelial cells, keratinocytes, and lymphocytes. Thus, the observed gene expression values are an average across all the cells and not fibroblasts only. Immunosuppressive drug therapy is another potential confounder although given the small sample size, we were unable to observe any relationship between the specific drugs and gene expression patterns. Notwithstanding these limitations, the major strength of this study is the homogeneity of the patient cohort with respect to the clinical phenotype of early active dcSSc and black ethnicity.

In conclusion, we observed dysregulation of several genes in the Wnt signaling pathway, many of which have not been reported previously. Furthermore, PCA showed two distinct patient clusters, which co-segregated with the presence or absence of clinical inflammatory features and may reflect different pathological pathways in SSc.

Acknowledgments We thank Dr. Frank Staedtler (Novartis, Basel) for providing us with the Human Wnt Signaling Pathway RT^2 Profiler PCR Array. MT is supported by the Medical Research Council of South Africa. MR is a South African Research Chair in Genomics and Bioinformatics of African populations hosted by the University of the Witwatersrand, funded by the Department of Science and Technology and administered by National Research Foundation of South Africa.

Funding information This work was funded by the Connective Tissue Diseases Grant through the University of the Witwatersrand Faculty of Health Sciences and the National Health Laboratory Service Research Grant. This project was funded by the CRG-Wits-Novartis Visiting Scientist Exchange Program.

Compliance with ethical standards

The study was approved by the Human Research Ethics Committee (Medical), Faculty of Health Sciences, University of the Witwatersrand, and all participants consented in accordance with the Declaration of Helsinki guidelines.

Disclosures None.

References

1. Bhattacharyya S, Wei J, Varga J (2012) Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol 8(1):42–54. https://doi.org/10.1038/nrrheum.2011.149
2. Beyer C, Dees C, Distler JH (2012) Morphogen pathways as molecular targets for the treatment of fibrosis in systemic sclerosis. Arch Dermatol Res. https://doi.org/10.1007/s00403-012-1304-7
3. Dees C, Zerr P, Tomcik M, Beyer C, Horn A, Akhmetshina A, Palumbo K, Reich N, Zwerina J, Sticherling M, Mattson MP, Distler O, Schett G, Distler JH (2011) Inhibition of Notch signaling prevents experimental fibrosis and induces regression of established

fibrosis. Arthritis Rheum 63(5):1396–1404. https://doi.org/10.1002/art.30254
4. Lam AP, Gottardi CJ (2011) Beta-catenin signaling: a novel mediator of fibrosis and potential therapeutic target. Curr Opin Rheumatol 23(6):562–567. https://doi.org/10.1097/BOR.0b013e32834b3309
5. Bergmann C, Distler JH (2016) Canonical Wnt signaling in systemic sclerosis. Lab Investig 96(2):151–155. https://doi.org/10.1038/labinvest.2015.154
6. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, McCalmont TH, Brown PO, Botstein D, Connolly MK (2003) Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A 100(21):12319–12324
7. Gardner H, Shearstone JR, Bandaru R, Crowell T, Lynes M, Trojanowska M, Pannu J, Smith E, Jablonska S, Blaszczyk M, Tan FK, Mayes MD (2006) Gene profiling of scleroderma skin reveals robust signatures of disease that are imperfectly reflected in the transcript profiles of explanted fibroblasts. Arthritis Rheum 54(6):1961–1973. https://doi.org/10.1002/art.21894
8. Akhmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, Horn A, Kireva T, Beyer C, Zwerina J, Schneider H, Sadowski A, Riener MO, MacDougald OA, Distler O, Schett G, Distler JH (2012) Activation of canonical Wnt signalling is required for TGF-beta-mediated fibrosis. Nat Commun 3:735. https://doi.org/10.1038/ncomms1734
9. Masi AT, Subcommittee For Scleroderma Criteria of the American Rheumatism Association D, Therapeutic Criteria C (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 23(5):581–590. https://doi.org/10.1002/art.1780230510
10. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA Jr, Rowell N, Wollheim F (1988) Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 15(2):202–205
11. Valentini G, Della Rossa A, Bombardieri S, Bencivelli W, Silman AJ, D'Angelo S, Cerinic MM, Belch JF, Black CM, Bruhlmann P, Czirjak L, De Luca A, Drosos AA, Ferri C, Gabrielli A, Giacomelli R, Hayem G, Inanc M, McHugh NJ, Nielsen H, Rosada M, Scorza R, Stork J, Sysa A, van den Hoogen FH, Vlachoyiannopoulos PJ (2001) European multicentre study to define disease activity criteria for systemic sclerosis. II Identification of disease activity variables and development of preliminary activity indexes. Ann Rheum Dis Ann Rheum Dis 60 (6):592–598
12. Cheng S-L, Shao J-S, Halstead LR, Distelhorst K, Sierra O, Towler DA (2010) Activation of vascular smooth muscle parathyroid hormone receptor inhibits Wnt/β-catenin signaling and aortic fibrosis in diabetic arteriosclerosis. Circ Res 107(2):271–282
13. Oda K, Yatera K, Izumi H, Ishimoto H, Yamada S, Nakao H, Hanaka T, Ogoshi T, Noguchi S, Mukae H (2016) Profibrotic role of WNT10A via TGF-beta signaling in idiopathic pulmonary fibrosis. Respir Res 17:39. https://doi.org/10.1186/s12931-016-0357-0
14. Spanjer AI, Baarsma HA, Oostenbrink LM, Jansen SR, Kuipers CC, Lindner M, Postma DS, Meurs H, Heijink IH, Gosens R (2016) TGF-β-induced profibrotic signaling is regulated in part by the WNT receptor Frizzled-8. FASEB J 30(5):1823–1835
15. He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y (2009) Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol 20(4):765–776. https://doi.org/10.1681/ASN.2008060566
16. Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F (2002) Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 22(4):1172–1183
17. Lawrence A, Khanna D, Misra R, Aggarwal A (2006) Increased expression of basic fibroblast growth factor in skin of patients with systemic sclerosis. Dermatol Online J 12 (1):2
18. Zuo F, Kaminski N, Eugui E, Allard J, Yakhini Z, Ben-Dor A, Lollini L, Morris D, Kim Y, DeLustro B (2002) Gene expression analysis reveals matrilysin as a key regulator of pulmonary fibrosis in mice and humans. Proc Natl Acad Sci 99(9):6292–6297
19. Milano A, Pendergrass SA, Sargent JL, George LK, McCalmont TH, Connolly MK, Whitfield ML (2008) Molecular subsets in the gene expression signatures of scleroderma skin. PLoS One 3(7):e2696
20. Bennett LB, Taylor KH, Arthur GL, Rahmatpanah FB, Hooshmand SI, Caldwell CW (2010) Epigenetic regulation of WNT signaling in chronic lymphocytic leukemia. Epigenomics 2(1):53–70. https://doi.org/10.2217/epi.09.43