Affected and non-affected skin fibroblasts from systemic sclerosis patients share a gene expression profile deviated from the one observed in healthy individuals

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Abstract

Objectives

To evaluate the gene expression profile of fibroblasts from affected and non-affected skin of systemic sclerosis (SSc) patients and from controls.

Materials and methods

Labeled cDNA from fibroblast cultures from forearm (affected) and axillary (non-affected) skin from six diffuse SSc patients, from three normal controls, and from MOLT-4/HEp-2/normal fibroblasts (reference pool) was probed in microarrays generated with 4193 human cDNAs from the IMAGE Consortium. Microarray images were converted into numerical data and gene expression was calculated as the ratio between fibroblast cDNA (Cy5) and reference pool cDNA (Cy3) data and analyzed by R environment/Aroma, Cluster, Tree View, and SAM softwares. Differential expression was confirmed by real time PCR for a set of selected genes.

Results

Eighty-eight genes were up- and 241 genes down-regulated in SSc fibroblasts. Gene expression correlation was strong between affected and non-affected fibroblast samples from the same patient ($r>0.8$), moderate among fibroblasts from all patients ($r=0.72$) and among fibroblasts from all controls ($r=0.70$), and modest among fibroblasts from patients and controls ($r=0.55$). The differential expression was confirmed by real time PCR for all selected genes.

Conclusions

Fibroblasts from affected and non-affected skin of SSc patients shared a similar abnormal gene expression profile, suggesting that the widespread molecular disturbance in SSc fibroblasts is more sensitive than histological and clinical alterations. Novel molecular elements potentially involved in SSc pathogenesis were identified.

Key words

Systemic sclerosis, gene expression, fibroblast, cDNA microarrays.

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Introduction

Systemic sclerosis (SSc) is a multi-organ inflammatory disease of unknown etiology characterized by extensive fibrosis of the skin and visceral organs (1). SSc fibroblasts exhibit accelerated proliferation rate and increased production of extracellular matrix components, such as type I, III, V, VI and VII collagens, fibronectin, and tenascin (2-5). Fibroblasts are also involved in the organization of the excessively produced extracellular matrix and in the communication with other cells that play an important role in the pathophysiology of the fibrotic alterations in SSc (6, 7). The study of fibrosis in SSc has been aided by previous observations that explanted fibroblasts are able to keep original phenotypic features for up to the tenth passage *in vitro* (8).

The application of the cDNA microarray technology to the study of gene expression by scleroderma fibroblasts has provided interesting information and made clear that such approach may be useful in the study of disease mechanisms in SSc (9-11). In 2001, Zhou *et al*. have shown that the genes for several SSc-relevant autoantigens were up-regulated in cultured SSc dermal fibroblasts from affected and non-affected skin (9). In 2003, Whitfield *et al*. analyzed microarrays for over 12.000 genes and found that affected and non-affected SSc whole skin displayed a similar gene expression pattern, which was different from the skin of non-affected individuals (10). More recently Tan *et al*. analyzed the expression profile for over 8,000 genes and observed that early passage dermal fibroblasts from non-affected SSc skin showed a different expression profile in relation to fibroblasts from non-affected individuals (11).

These findings are exciting in indicating that the molecular pathologic pattern of SSc is established in fibroblasts from non-affected skin. However, it is also possible that a putative differential expression of a specific set of genes in fibroblasts from affected skin in relation to non-affected skin is overshadowed by the massive similarity in the overall individual gene expression profile and might not be detected depending on the gene panel studied. In the present study we sought to check this possibility by targetting an independent set of genes with the cDNA microarray technology to assess the differential gene expression in early-passage explanted fibroblasts from affected and non-affected skin from patients with diffuse SSc as well as from healthy controls. In addition, gene expression sample normalization was performed using a reference RNA pool from three different cell sources in order to improve the accuracy of estimation of possible gene differential expression.

Material and methods

Patients meeting the American College of Rheumatology criteria for classification as diffuse SSc (12) (2 women and 4 men) aged 22 to 46 years old were recruited from the Outpatient Clinic at Universidade Federal de São Paulo. Controls were healthy adults (1 woman and 2 men) aged 32 to 46 years old, who volunteered for donation of the samples. All participants signed an informed consent form approved by the Ethics Committee.

Primary human dermal fibroblast cultures were established by outgrowth of 5mm-wide punch biopsy specimens from the distal dorsal forearm (affected skin) and axillary region (non-affected skin) from SSc patients and healthy volunteers. Rodnan score (13) at the site of biopsy was 2 to 3 for affected and 0 for non-affected skin. Cultures were established in DMEM containing 10% fetal bovine serum, penicillin 10.000UI/mL, and streptomycin 10.000μg/mL at 5% CO_2 and 37°C. Total RNA was extracted from fibroblast cultures at the third passage using the Trizol® reagent (Invitrogen, Carlsbad, CA, USA). The integrity of RNA samples was evaluated by denaturing agarose gel electrophoresis under standard conditions and controlled for protein and phenol contamination by UV spectrophotometry.

The gene expression profile of fibroblasts was assessed using glass slide cDNA microarrays containing 4.193 sequences in replicate from the human expressed sequence tags (ESTs) cDNA library from the "International Molecular

Analysis of Genomes and their Expression" - IMAGE Consortium, (14) gently provided by Dr. Catherine Nguyen from Unité INSERM ERM 206, Marseille, France. Microarrays were constructed in our laboratory on type 7 mirrored glass slides (Amersham Biosciences – Molecular Dynamics-Sunny Valley, CA, USA) using a Generation III array spotter (Amersham Biosciences) and cross-linked with ultraviolet cross-linker. Microarrays were prepared based on published protocols using 0.75-1.0 kb PCR products from the cDNA clones (15).

The cDNA samples were prepared from 10μg total RNA from each fibroblast culture, using CyScribe post-labeling kit (Amersham Biosciences) and oligo dT12-18 plus random primers for reverse transcription reaction. Reverse transcription was performed in the presence of aminoallyl nucleotide triphosphates to generate aminoallyl cDNA. After purification in GFX column (Amersham Biosciences) the aminoallyl cDNA was labeled with NHS ester of Cy5 dye and purified again in GFX column. A cDNA pool originated from equimolar amounts of total RNA from three different cells lines [HEp-2 (human larynx epithelial carcinoma), MOLT-4 (human lymphoblast T cells), and normal human dermal fibroblasts] was used as a reference for comparison of patients and controls samples. The aminoallyl cDNA pool was labeled as above using the Cy3 dye and was able to successfully hybridize over 70% of the microarray panel used.

Hybridization and washing were performed for a 15-hour period in a Lucidea Automated Slide Processor-ASP (Amersham Biosciences) and the microarray slides scanned in Generation III laser scanner (Amersham Biosciences). The quality control of the spots and the acquisition of digital data were performed with the Spotfinder software (http://www.tigr.org/software).

The quality control criteria evaluated if the size, contour and saturation of each spot were adequate. Another criterion (1) was that the spot should present intensity above two backgrounds plus two standard deviations. The normalization was performed by Print-tips Lowess and Scale methods using R environment (www.r-project.org) with Aroma package (www.maths.lth.se/help/R/aroma) (16-18). Cluster analysis among genes and patients was performed using Cluster software (http://rana.lbl.gov/EisenSoftware.htm) and the results were visualized by means of the Java Tree View software (http://genetics.stanford.edu/~alok/TreeView) (19). SAM (Significance Analysis of Microarray) software (http://www-stat.stanford.edu/~tibs/SAM/) was used to find the genes with statistically significant differential expression (20).

For quantitative real-time PCR, total RNA (5μg) of each sample was treated with 5U DNase I (Invitrogen) and subjected to reverse transcription with Superscript III (Invitrogen). Real-time PCR was performed in ABI 7000 (Applied Biosystems, Foster City, CA) and signal detection was obtained with the Sybr Green reagent (Applied Biosystems). The cDNA (1μL) was added to 7.5μL master mix Sybr Green, 0.5μL of forward and reverse primers (18uM), and Milli Q water qsp 15μL. The thermocycling protocol was 10 minutes at 50°C, 5 minutes at 95°C, and 45 cycles with 30 seconds at 95°C and 1 minute at 60°C. Amplification of a single PCR product was confirmed by gel electrophoresis and melting dissociation curve analysis. The amount of mRNA in the sample was expressed as the relative amount to the GAPDH and β-actin genes, according to the formula $2^{-\Delta CT}$, where ΔCT is CT_{gene}-$CT_{housekeeping}$ (21, 22). The primers used were designed according to the Primer express software (Applied Biosystems): SPA-1 (Signal-Induced Proliferation Associated Gene-1): 5´CCCCTGCACTGATAACGTCTGT3´ and 5´AGACGTCGGATTTGCGGAA3´; VG5Q (Vascular Growth 5 Quest gene): 5´TGAAAACGCCGATCCAGCT3´ and 5´AAACCGCTCTCGTGCTTTGTC; MMP9 (matrix metalloproteinase 9) gene: 5´ACGACGT CTTCCAGTACCGAGA and 5´TAGGTCACGTAGCCCACTTGGT 3´; Kinesin C (KIF1C) gene: 5´TCGACTGGAACTTTGCCCA3' and 5´CCGGTACTGATTCTCCAGATCC 3´; IK cytokine gene: 5´ACTGATGGAAAAGCCCCAGAA3' and 5´CATTCCGCTCATATGCTTTGC 3´; PSG-1 (pregnancy-specific glycoprotein-1) gene: 5´AGCAGCAACTTAAACCCCAGG 3´ and 5´GCTCTGACCATTCATCCACCA 3; GAPDH (glyceraldehyde- 3-phosphate dehydrogenase) gene: 5´TCGGAGTCAACGGATTTGG and 5´GATGGCAACAATATCCACTTTACCA 3´; β-actin gene: 5´-TAATGTCACGCACGATTTCCC-3' and 5´-TCACCGAGCGCGGCT-3'.

Table I. Synoptic characterization of the six patients with diffuse systemic sclerosis.

Patients	Age/ gender/ ethnicity	Duration (years)	TSS*	Local skin score**	Anti-Scl-70	Involved organs	Medication
P1	44/F/C	2	33	2	P	Lung, GI	Xylocaine, nifedipine
P2	30/M/A	4	22	2	NA***	Lung	Xylocaine /DPA
P3	34/M/C	7	24	3	N	Lung, GI	Xylocaine, cyclophosphamide
P4	35/F/A	5	31	2	P	GI, Heart	Cyclophosphamide
P5	60/M/C	3	31	3	N	Lung, GI, Heart	Xylocaine, captopril, cyclophosphamide, corticosteroid
P6	42/M/A	3	46	3	N	Lung, GI, Heart	Xylocaine, cyclophosphamide

*TSS: Rodnan's modified skin score.
**Skin score at the site of biopsy for affected skin.
F: female; M: male; C: Caucasoid; A: Afro-descendant; P: positive; N: negative; NA: not available; GI: gastrointestinal tract; DPA: D-penicillamine.

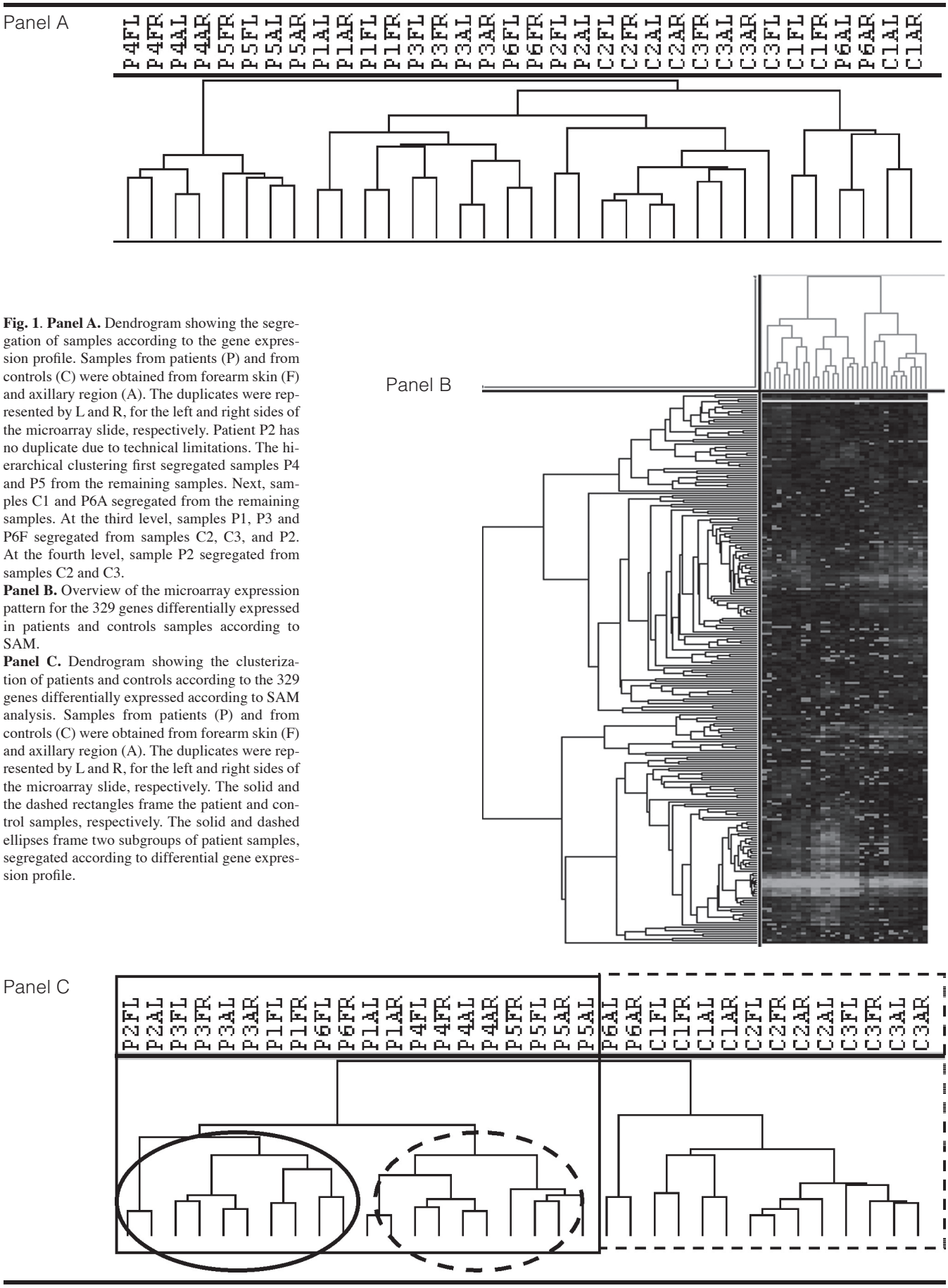

Fig. 1. **Panel A.** Dendrogram showing the segregation of samples according to the gene expression profile. Samples from patients (P) and from controls (C) were obtained from forearm skin (F) and axillary region (A). The duplicates were represented by L and R, for the left and right sides of the microarray slide, respectively. Patient P2 has no duplicate due to technical limitations. The hierarchical clustering first segregated samples P4 and P5 from the remaining samples. Next, samples C1 and P6A segregated from the remaining samples. At the third level, samples P1, P3 and P6F segregated from samples C2, C3, and P2. At the fourth level, sample P2 segregated from samples C2 and C3.

Panel B. Overview of the microarray expression pattern for the 329 genes differentially expressed in patients and controls samples according to SAM.

Panel C. Dendrogram showing the clusterization of patients and controls according to the 329 genes differentially expressed according to SAM analysis. Samples from patients (P) and from controls (C) were obtained from forearm skin (F) and axillary region (A). The duplicates were represented by L and R, for the left and right sides of the microarray slide, respectively. The solid and the dashed rectangles frame the patient and control samples, respectively. The solid and dashed ellipses frame two subgroups of patient samples, segregated according to differential gene expression profile.

Results

The study group comprised six patients with diffuse SSc (Table I) and three normal controls, rendering twelve samples from SSc patients (six affected and six non-affected samples) and six from normal controls (three from the forearm and three from the axillary region).

Among the originally tested 4193 genes, the quality control criteria approved the data for 2632 genes. Hierarchical clustering of all samples according to the expression profile of these genes (Fig. 1A) showed that duplicates segregated together ($r \geq 0.8$), what argues for the good reproducibility of the assay. Affected and non-affected skin of the same individual showed a correlation score equal or above 0.7. This good concordance rate was supported by SAM analysis that could not detect a single gene differentially expressed between affected and non-affected skin in the same patient (data not shown). The hierarchical clustering showed a trend for segregation of samples from patients and controls. Genes differentially expressed between patient and control groups were identified with the assistance of the SAM software. For this analysis, samples from affected and non-affected skin from patients were put together in one group since they did not differ from each other in the previous analysis. The overall comparison showed 329 genes differentially expressed between patient and control groups with a false discovery rate (FDR) of 8.70. Among these, 88 were up-regulated (Table II) and 241 were down-regulated (Table III).

A second round of hierarchical clustering was performed based on the analysis of the expression profile of the 329 genes differentially expressed according to SAM analysis (Figs. 1B and C). There was an excellent correlation for duplicate samples ($r > 0.9$) and for affected and non-affected skin samples in the same patient ($0.9 > r > 0.8$). As expected, the segregation of patients and controls was even better defined and only one patient sample (P6A, non-affected skin) segregated together with the samples of the control group (Fig. 1C). A good correlation was found for samples of the patient group ($r = 0.72$)

Table II. Top twenty up-regulated genes in dermal fibroblasts from systemic sclerosis patients.

Clone ID	Symbol	Name
IMAGE:26940	SIPA1L3	Signal-induced proliferation-associated 1 like 3
IMAGE:26608	LOC54103	Hypothetical protein LOC54103
IMAGE:135010	LOC653464	Similar to SLIT-ROBO Rho GTPase-activating protein 2 (srGAP2) (Formin-binding protein 2)
IMAGE:24463	C13orf27	Chromosome 13 open reading frame 27
IMAGE:133114	PRSS23	In multiple clusters
IMAGE:258454	BAG4	BCL2-associated athanogene 4
IMAGE:27896	MATR3	Matrin 3
IMAGE:258966	RPS6KA5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5
IMAGE:250434	POLH	Polymerase (DNA directed), eta
IMAGE:25081	C9orf97	Chromosome 9 open reading frame 97
IMAGE:28574	DLEC1	Acetyl-Coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-Coenzyme A thiolase)
IMAGE:142244	MAML3	Mastermind-like 3 (Drosophila)
IMAGE:258072	PDIK1L	PDLIM1 interacting kinase 1 like
IMAGE:188350	DDX59	DEAD (Asp-Glu-Ala-Asp) box polypeptide 59
IMAGE:142556	PSG2	Pregnancy specific beta-1-glycoprotein 2
IMAGE:26940	SIPA1L3	Signal-induced proliferation-associated 1 like 3
IMAGE:26608	LOC54103	Hypothetical protein LOC54103
IMAGE:135010	LOC653464	Similar to SLIT-ROBO Rho GTPase-activating protein 2 (srGAP2) (Formin-binding protein 2)
IMAGE:27769	CXCL14	Chemokine (C-X-C motif) ligand 14
IMAGE:262278	KIF1C	Kinesin family member 1C

Table III. Top twenty down-regulated genes in dermal fibroblasts from systemic sclerosis patients.

Clone ID	Symbol	Name
IMAGE:23334	CCNYL1	Cyclin Y-like 1
IMAGE:21844	ARL4C	ADP-ribosylation factor-like 4C
IMAGE:21834	PPP2R5E	Protein phosphatase 2, regulatory subunit B', epsilon isoform
IMAGE:22177	TBC1D22B	TBC1 domain family, member 22B
IMAGE:26736	C20orf198	Chromosome 20 open reading frame 198
IMAGE:23397	ZMYND19	Zinc finger, MYND-type containing 19
IMAGE:23792	RXRA	Retinoid X receptor, alpha
IMAGE:24519	PNPLA2	Patatin-like phospholipase domain containing 2
IMAGE:23143	KIF13A	Kinesin family member 13A
IMAGE:33136	LOC388889	Hypothetical LOC388889
IMAGE:22040	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
IMAGE:135766	LOC285500	Hypothetical protein LOC285500
IMAGE:27080	ZFAND6	Zinc finger, AN1-type domain 6
IMAGE:22279		Transcribed locus
IMAGE:21627	R3HDM1	R3H domain containing 1
IMAGE:136508	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
IMAGE:21643	AKAP7	A kinase (PRKA) anchor protein 7
IMAGE:23282	IK	IK cytokine, down-regulator of HLA II
IMAGE:24506	AGGF1	Angiogenic factor with G patch and FHA domains 1 (VG5Q)
IMAGE:136318	MFHAS1	Malignant fibrous histiocytoma amplified sequence 1

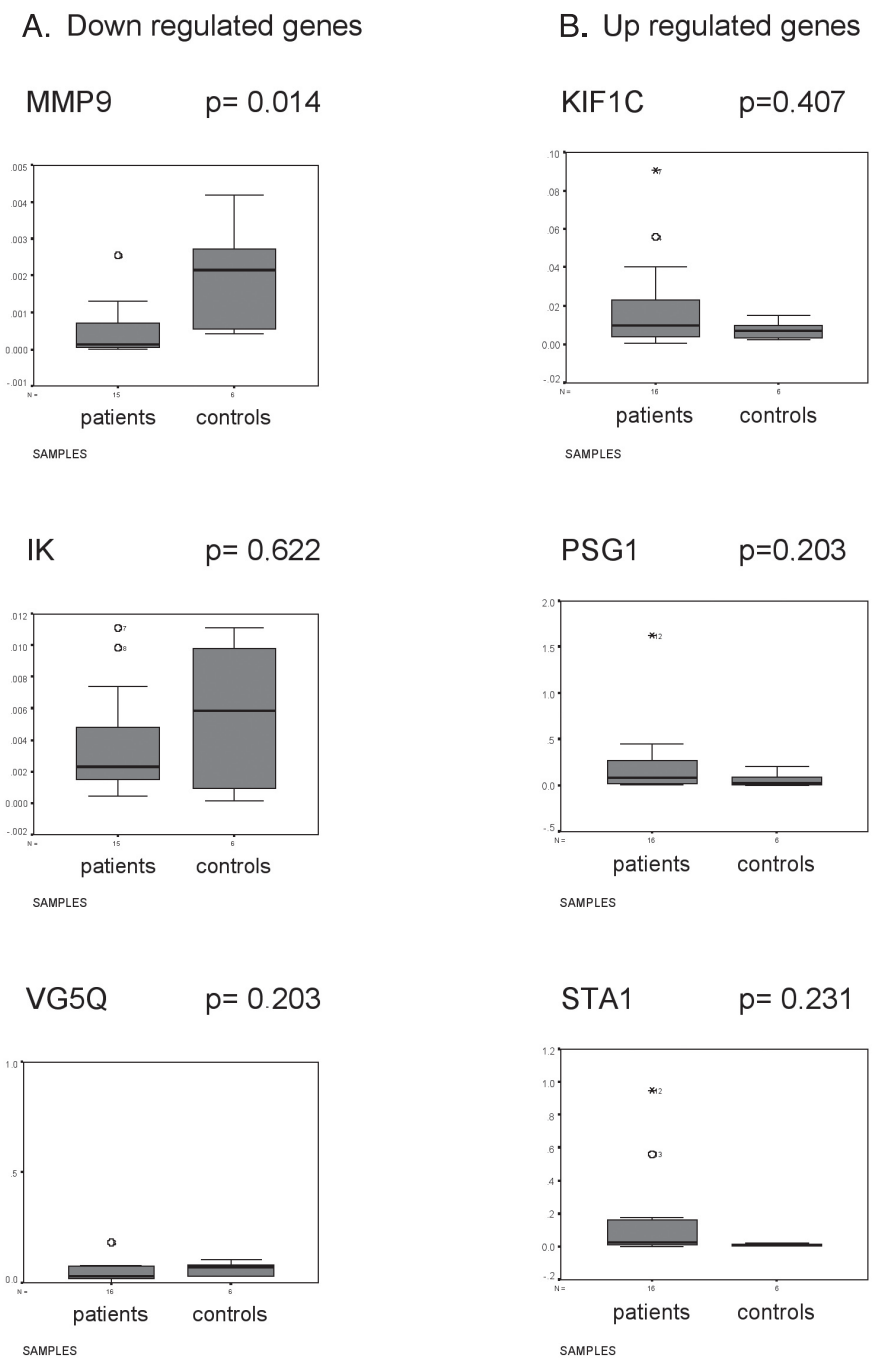

Fig. 2. Box-plot graph of the relative gene expression assessed by real-time PCR with regard to the expression of the housekeeping gene GAPDH. The upper and lower horizontal bars represent the highest and lowest figures in each group The rectangle represents 75% of the samples and the upper and lower limits of the rectangle represent the 75th and 25th percentile, respectively. The thick horizontal bar represents the median.

and for samples in the control group (r=0.70). The overall correlation of all samples was modest (r=0.55). The cluster analysis within the patient group could segregate two subgroups, one comprising samples P2, P3, P1F and P6F (Fig. 1C, solid ellipsis), and the other comprising samples P1A, P4 and P5 (Fig. 1C, dashed ellipsis). No particular clinical or laboratory feature was specifically associated with any of these two subgroups.

Genes differentially expressed were subjected to gene ontology (GO) analysis in order to annotate the cellular processes preferentially stimulated or repressed in SSc fibroblasts. The analysis using the Web interface FatiGO (www.fatigo.org) at level 4 showed 57 classes involved. Some genes were represented in 2 or more classes. Most of the genes differentially expressed belonged to classes GO:0006810 (transport), GO:0007165 (signal transduction), GO:0019222 (regulation of metabolism), GO:0043170 (macromolecule metabolism), GO:0051244 (regulation of cellular physiological process), GO:0044237 (cellular metabolism), and GO:0044238 (primary metabolism). Among up-regulated genes, the most prominent classes were GO:0009306 (protein secretion), GO:0016049 (cell growth), and GO:0001775 (cell activation). For down-regulated genes, the most represented classes were GO:0001944 (vasculature development), GO:0007155 (cell adhesion), and GO:0030155 (regulation of cell adhesion). Real Time PCR (RT-PCR) was used to study six of the genes found to be differentially expressed in SSc fibroblasts by the microarray methodology. According to microarray experiments three of these were found to be up-regulated: Signal-Induced Proliferation-Associated Gene-1 (SPA-1), Pregnancy-Specific Glycoprotein-1 (PSG-1), and Kinesin 1C (KIF1C). The other three were down-regulated: Vascular Growth 5 Quest (VG5Q), Metalloproteinase-9 (MMP9), and Interleukin IK. Real Time PCR data for these six genes obtained with the GAPDH (Fig. 2) and β-actin (data not shown) as reference standards were concordant with those obtained in the microarray platform, although the magnitude of the relative expression varied between the two methods.

Discussion

In the present study we sought to analyze the gene expression profile of dermal fibroblasts from SSc patients in order to gain some insight into the behavior of these cells so intimately involved in the pathophysiology of this disease. Among 4,193 genes randomly selected from a general human cDNA library, three hundred and twenty-nine genes were found to be differentially expressed in SSc fibroblasts as compared to normal fibroblasts. The hierarchical clustering of all samples according to

the expression profile of all analyzed genes clearly demonstrated a high correlation between the gene expression profile of affected and non-affected skin fibroblasts. Annotation according to the Gene Ontology Classification showed that most of the differentially expressed genes belonged to metabolic pathways potentially relevant to the disease pathophysiology. The highest gene expression correlation was observed for samples from affected and non-affected skin from the same patient, followed by the moderate correlation between samples from different individuals in the same group. The lowest correlation was observed for the overall comparison of SSc patients and controls, supporting the notion that dermal fibroblasts from affected and non-affected SSc skin do present a significantly skewed gene expression profile in relation to those from normal individuals.

The finding of a different gene expression profile in SSc dermal fibroblasts is expected from the clearly altered phenotype of these cells. In fact, changes in the gene expression of type I, III, IV and VI collagens, decorin, and MMPs in dermal fibroblast from SSc patients have been previously demonstrated by early Northern blot analysis and recent microarray analysis. (9, 11, 23) In contrast, the observed similarity in the gene expression profile among fibroblasts from affected and non-affected skin from SSc patients is in apparent contradiction with the clearly different phenotype of the tissue from where these cells were originally obtained. This intriguing finding is supported by previous microarray studies with RNA from skin fibroblasts (9, 11, 24) and total skin from SSc patients (10) using independent panels of genes and different normalization strategies. The altered gene expression profile in fibroblasts from non-affected skin may represent a necessary but not sufficient condition for the development of the SSc phenotype. It can be anticipated that several other steps must be accomplished in order to fulfill the complete expression of the final SSc phenotype and these may include post-transcription, translation, and post-translation processes. The altered gene expression profile observed in non-affected fibroblasts may be conditioned by genetic predisposition to disease. This interpretation is supported by the recent finding that 40-50% of monozygotic twins clinically discordant for SSc show concordance for fibroblast gene expression profile (24).

Studies on gene expression frequently face a dilemma with regard to sample selection (25). Original tissue samples provide cells with the closest to the real gene expression pattern, but most tissues contain a mixture of several cell types, what makes it difficult to interpret the obtained data provided that each cell type has a peculiar gene expression pattern. The alternative of studying primary cultures of a specific cell type from the tissues of interest has the potential caveat that the original phenotype and the corresponding gene expression pattern may not be maintained in cultured cells. In the case of SSc fibroblasts, however, the deviation from the original gene expression pattern during *in vitro* culturing is probably less marked since these cells are known to keep several phenotypic features for several passages (8, 25). Notwithstanding a recent study by Gardner *et al*. showed that dermal fibroblasts in primary culture display a smaller number of differentially expressed genes as compared to fibroblasts from biopsy samples (26). This observation suggests that the existing *in vivo* milieu may contribute to the altered gene expression observed in SSc dermal fibroblasts.

The methodology used in the several studies (9-11, 24) and in the present one has many common aspects and a few differences related to the panel of genes analyzed, the source of mRNA, and the data analysis strategy. These studies have observed an altered gene expression profile in affected and non-affected SSc dermal fibroblasts and the sets of differentially expressed genes pointed in each study are conditioned by the panel of genes analyzed. The findings herein obtained with the microarray technique were confirmed by real time PCR with regard to 6 genes selected according to the magnitude and consistency of the microarray differential expression in the various SSc samples. Pregnancy-specific glycoprotein-1 (PSG1) gene, found to be up-regulated in SSc dermal fibroblasts, induces the expression of IL-6, IL-10, and TGF-β1 in monocytes (27-29) and thus may contribute to the Th2 phenotype and to the inhibition of IL-12, IFN-γ, and TNF-α production observed in SSc (30, 31). Signal-induced proliferation-associated gene-1 (SPA-1) transcribes STA-1, a GAP (GTPase-activating protein) that inactivates RAP1GTP. RAP1GTP can inhibit proliferation, acting in antagonism with RAS gene in some cell types (32). The present finding of an up-regulation in STA-1 expression may override the inhibitory effect of RAP1GTP in SSc dermal fibroblasts and thus contribute to the observed proliferative phenotype. Kinesin member 1C, also shown to be up-regulated in SSc fibroblasts, is involved in vesicle transport from the Golgi complex to the endoplasmic reticulum (33, 34) and thus may play a relevant role in the increased secretion of extracellular matrix components by the dermal fibroblasts in SSc. Vascular Growth 5 Quest (VG5Q) stimulates vascular growth and proliferation in the neovascularization process (35). The demonstration of down-regulation of VG5Q in SSc dermal fibroblasts may cast some light into the understanding of the extensive devascularization process regularly observed in this disease. Metalloproteinase-9 (MMP9) degrades type I, III, IV and V collagens, proteoglycans, and elastin. The observed down-regulation of MMP9 gene expression in SSc dermal fibroblasts would be consistent with a putative decrease in matrix remodeling, thus contributing to the characteristic fibrotic phenotype of this disease. In fact, Kikuri *et al*. have previously found that MMP9 activity was diminished in the serum of SSc patients (36). On the other hand, Wan-Uk Kim *et al*. found it to be elevated in the serum of SSc patients and in the supernatant of SSc dermal fibroblast cultures stimulated by TGF-β, IL-1 or TNF-α (37). Cytokine IK inhibits IFNγ-induced expression of Major Histocompatibility Complex (MHC) class II genes by counteracting the positive effect of CIITA (class II transactivator)

in both inducible and constitutive MHC class II regulation pathways (38, 39). Dermal fibroblasts and endothelial cells in SSc display an aberrantly increased MHC class II expression, especially HLA-DR (40). This is consistent with the present finding of down-regulated expression of Cytokine IK in SSc dermal fibroblasts, which may skew the CIITA/IK ratio towards an increased expression of HLA-DR molecules. The connection between SSc phenotype and the above mentioned molecular pathways appears to support the differential expression of these six genes in SSc dermal fibroblasts and therefore warrant further studies to analyze the related protein expression in tissue and cultured SSc dermal fibroblasts.

In conclusion, the present study has originally disclosed several differentially expressed genes between dermal fibroblasts from SSc patients and normal controls, some of which deserve future study since the imbalance in their expression finds resonance with the pathophysiology of SSc. In addition, using a panel of genes distinct from other investigators, we confirmed the following previous observations (9-11, 24, 26) on gene expression of SSc fibroblasts: 1) dermal fibroblasts from SSc patients present a differential gene expression pattern in relation to dermal fibroblasts from healthy controls; 2) affected and non-affected SSc dermal fibroblasts show a similar gene expression pattern. The latter observation suggests that extensive changes in gene expression are already present in apparently uninvolved fibroblasts and herald yet unknown down-stream events that ultimately will cause the frank fibrotic SSc phenotype. The concordance of previous (9-11, 24, 26) and present findings corroborate the consistency of the observed phenomena and of the microarray technology.

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