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Skin gene expression is prognostic for the trajectory of skin disease in patients with diffuse cutaneous systemic sclerosis

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Abstract

Objective: Currently there are no clinical or laboratory measures that accurately predict progression of skin fibrosis and organ involvement in patients with systemic sclerosis (SSc). The goal of this study is to identify skin biomarkers in early diffuse cutaneous SSc patients, to prognosticate the progression of skin fibrosis.

Methods: We analyzed clinical data and skin biopsy gene expression from 38 placebo patients, part of the Roche faSScinate phase 2 study of tocilizumab in SSc. RNA samples were analyzed using nCounter technology. A trajectory model, based on the modified Rodnan skin score was used to describe three skin disease trajectories over time. We examined the association of skin gene expression with trajectory groups of skin score using the Chi-square test. We used logistic regression to examine the prognostic power of each gene identified.

Results: We found that placebo treated patients with high CD14, SERPINE1, IL13RA1, CTGF, and OSMR mRNA expression at baseline were more likely to have progressive skin score trajectories. We also found that those genes were prognostic for the risk of skin progression and IL13RA1, OSMR and SERPINE 1 performed the best.

Conclusion: Skin gene expression of biomarkers associated with macrophages (CD14, IL13RA1) and TGFβ activation (SERPINE1, CTGF, OSMR) are prognostic for progressive skin disease. These biomarkers might help to guide decisions about which patients should be considered for aggressive therapies and/or for clinical trials.

Keywords: Systemic sclerosis, skin gene expression, prognostic biomarkers.

Short Title: Prognostic biomarkers in diffuse cutaneous systemic sclerosis

Introduction

Currently there are no clinical or laboratory measures that accurately predict progression of skin fibrosis and organ involvement in patients with SSc. Several studies, including retrospective cohort analyses and randomized clinical trials, have shown that the severity of skin fibrosis, as assessed by MRSS, is predictive of disease mortality [1] [2]. In particular, Shand et al. defined three distinct skin score trajectory subgroups,

using latent variable modeling, and showed that patients with the worst skin score trajectory have significantly increased mortality [3].

Several clinical and serological measures have been associated with progressive skin disease. It is generally accepted that the fastest rates of skin disease progression are recorded early in the disease [4]. A recent observational study from the EUSTAR has shown that joint synovitis, short disease duration (less than 15 months) and low MRSS at baseline predict more progressive skin fibrosis [5]. Anti-RNA-polymerase III (anti-Pol3) is associated with scleroderma renal crisis (SRC) and more severe skin disease, though less interstitial lung disease (ILD) [4] [6]. Despite these findings there remains no broadly accepted methodology for assessing the likelihood of progressive skin disease and no validated prognostic biomarkers of skin disease evolution, limiting patient risk-stratification and consequently the ability to select patients with progressive disease for innovative therapies.

We recently reported that CD14 expression correlates strongly with progressive skin disease [7]. Using data collected from the faSScinate study, an international trial of tocilizumab (TCZ) in SSc [8], we describe trajectory patterns of skin score change over time using a group-based modeling approach in placebo (PBO) treated patients and assessed several potential prognostic biomarkers associated with these skin score changes. In addition, we also examined the relation of each biomarker to the change in skin scoreover time.

Methods

Study design and participants

Samples and clinical data for the discovery cohort used in this study were part of the faSScinate phase 2 study (ClinicalTrials.gov: NCT01532869). Briefly, the FaSScinate study was a randomized, double-blind, placebo-controlled phase 2 study of tocilizumab (162 mg subcutaneously weekly) in systemic sclerosis patients aged 18-year or older, with progressive disease of less than 5 years' duration since their first non-Raynaud's sign or symptom. In the current analysis, we focused on the FaSScinate patients treated with placebo (PBO). Of these (n=44) we excluded six subjects. Two patients did not have a biopsy at baseline. One patient had only two MRSS values (at baseline and at 8

weeks) and discontinued the study at week 16. The other three excluded patients had a drop of MRSS of greater than 12 units in two sequential assessments, suggesting that the scoring MRSS trajectory in these patients would be unreliable.

Skin biopsy gene expression analysis

RNA samples, used for analysis of gene expression, were analyzed using nCounter technology (NanoString Technologies, Seattle, WA, USA). Expression of genes was normalized to 12 housekeeping genes. Of the 83 genes selected for confirmation expression analysis, 62 transcripts were significantly overexpressed and two were significantly underexpressed in SSc patients compared with healthy controls (t-test, Bonferroni correction for multiple comparisons). Microarray data from the faSScinate trial, used for selecting prognostic genes, has been deposited in NCBI GEO, ID# GSE106358.

Statistical analysis

We described patterns of skin score change over time using a semiparametric mixture model [9]. Specifically, the distinctive trajectories of skin score were derived by modeling skin score as a function of time, i.e. the number of days in the study using a SAS Macro (PROC TRAJ) [10]. We assumed each trajectory of skin score had a linear pattern of decline and tested this assumption by including a quadratic term (i.e., testing for the possibility that change in skin score has a curved shape) and evaluated statistical significance of these terms for each trajectory group. Linear but not quadratic model terms were statistically significant ($p < 0.05$); thus we only included a linear term in our final models. The probability of each trajectory membership for a subject was estimated from the group-based model. Each subject was assigned to a specific trajectory group that had the highest estimated probability (i.e., posterior probability) compared with those of other trajectory groups. We used Bayesian information criteria (i.e., BIC) and Entropy (i.e., amount of classification error indexed by average posterior probability) to assess the model fit. In general, models with lower BIC values provide a better fit to the data, and Entropy statistics near 1 (above 0.8) convey a model with well-separated trajectories [9].

We divided expression of each gene into tertile groups. For expression of each gene we examined its association with trajectory groups of skin score using the Chi-square test. In addition, we examined the association between each gene expression at baseline and skin score change over time from baseline using Generalized-Estimating Equations in SAS with the "exchange" option for the working correlation matrix. In the regression model, the lowest tertile of each gene expression measure was used as the referent group to test the difference in change in MRSS score. Finally, we collapsed regressive and stable trajectories into one group and modeled the predictive ability of each gene by logistic regression. Using SAS, we assessed the predictive ability of the model according to discrimination and calibration. Discrimination was assessed using area under the curve (AUC), with the guidelines suggesting that values of at least 0.70 are needed for adequate prediction. Calibration was assessed using the Hosmer and Lemeshow test [11], where a significant result indicates poor calibration. Pearson correlations were calculated using Prism software (GraphPad Software, Inc.). Differences were considered significant at a P-value <0.05.

Results

Study patients

All patients enrolled in the faSScinate study met the 1980 American College of Rheumatology criteria for SSc, had active SSc of≤5 years disease duration since their first non-Raynaud symptom, and a MRSS between 15 to 40. In addition, at screening, active progressive disease of <1 year's duration was required—increase of≥3 MRSS units, involvement of one new body area with increase in MRSS ≥2 units or two new body areas with increase in MRSS ≥1 unit, other documentation of worsening skin thickening in the previous 6 months, or≥1 tendon friction rub plus ≥1 laboratory criterion (C-reactive protein [CRP]≥10·0 mg/L, erythrocyte sedimentation rate ≥28 mm/h, or platelets ≥330×1000/μL) [8]. The discovery cohort for the identification of prognostic biomarkers consisted of 38 patients from the PBO group (Table 1). For validation we studied microarray gene expression data from a second cohort of patients with diffuse cutaneous SSc (dcSSc, 20 patients in total). This group of 20 patients, which we have defined as the validation cohort, has similar clinical features to the discovery group. All the patients had diffuse cutaneous systemic sclerosis, and they are in their early phase

of the disease (less than 5 years from the first non Raynaud's phenomenon symptom). However, patients in the validation group had some significant differences compared with those in the discovery group. First, patients in the validation group were treated with immunosuppressant drugs, whereas the patients from the discovery group received only placebo during the study. Second, all patients in the validation group had only two measurements of MRSS, one at baseline and another at 24 weeks. Third, patients in the discovery cohort met certain additional criteria to define disease activity, whereas those in the validation cohort did not [12].

Gene expression and correlation with MRSS

Microarray data generated as part of the clinical trial from mid-forearm skin biopsies were analyzed for genes that correlated most highly with the change in skin score from baseline to six months after treatment with placebo. From a microarray that was generated as part of the faSScinate study, we selected 83 genes that highly correlated with the change in skin score at 6 months (Supplementary Figure 1). 62 of these were over expressed in SSc patients compared to the healthy control. Most of these genes correlating most highly with the change in MRSS were in gene clusters identifiable as part of TGFβ/profibrotic-, IL6/STAT3-, or IFN-pathways; or associated with macrophages. Other genes of interest were also included in the nCounter panel, as described previously [8]. Gene expression from each of the patients was tested using Counter technology (Nanostring Technologies, Inc.) as described previously [8].

Using this gene expression data, we calculated the correlation coefficient (r) in 34 of 38 placebo treated patients between gene expression at baseline and the change in MRSS at week 16 (4 patients were not included because of missing values at 16 weeks and/or at baseline). Based on this correlation, we clustered all the genes for which $|r| > 0.2$ (Figure 1). By inspection these genes grouped into three different clusters. Two of the clusters (group A and group B) contained many recognizable genes based on known biological relevance: TGF-β/profibrotic genes (Figure 1; group A) and macrophage-associated genes (Figure 1; group B). The third cluster contained genes without evident biological relationship (Figure 1; group C).

We compared the correlation between baseline gene expression and the change

in MRSS in this cohort of patients, defined as the discovery group, with microarray data from a second cohort of dcSSc patients defined as the validation cohort. We found that the correlation coefficients had the same trend for many of the genes in both cohorts (Supplementary Table 1), even though the R-values were different between the two groups. This might be due to differences in clinical features between the two groups, as well as the two different methods used for the gene expression analyses (nanostring versus microarray).

From the group of the genes that we identified (Supplementary table 1) we chose seven genes for further analysis based on the strength of correlation between baseline gene expression and the change in MRSS in both discovery and validation cohorts of >|0.2|. Since expression of many of the genes correlated highly with each other, we limited our analysis of co-regulated genes to the genes showing the largest correlation coefficients. Notably expression levels of CD14, chemokine (C-C motif) ligand 2 (CCL2), CD163, Macrophage Scavenger Receptor 1 (MSR1) and membrane-spanning 4-domains, subfamily a, member 4a (MS4A4A) were highly correlated and therefore we chose to focus on CD14 only. The following genes were analyzed further: CD14, interleukin 13 Receptor Alpha 1 (IL13RA1), SERPINE1, ONCOSTATIN M Receptor (OSMR), Connective Tissue Growth Factor (CTGF), Insulin Like Growth Factor Binding Protein 2 (IGFBP-2), and Interferon Regulatory Factor 7 (IRF7) (Table 2). All these genes were overexpressed in SSc patient skin compared to the healthy controls (Figure 1).

Descriptive Trajectory Data

As shown in Figure 2 we identified three trajectories of skin score change (y) over 48 weeks: 12 (30%) showed a regressive trajectory (regressive patients: y=17.69773-0.12504* weeks), 18 (45%) a stable trajectory (stable patients: y=25.02055 -0.08435* weeks), and 10 (25%) a progressive trajectory (progressive patients: y= 31.12353+0.11507* weeks). The progressive trajectory group started with a higher average MRSS (30.65) than the other two trajectories and the average skin score increased 17.7% at the end of 48 weeks. The regressive group started with a lower average MRSS (19.93), which decreased 33.9% at the end of follow up period. The

stable group started with an intermediate average MRSS (26.12) and experienced a slight decline in MRSS (16.2%) over the same time period. The average posterior probability of allocating study participants into trajectories (i.e., entropy) was $\geq$0.97, indicating an excellent precision that individuals were assigned to their most likely trajectories.

Association of Gene expression and Pattern of skin score progression

We examined the seven genes selected from the cluster groups (CD14, IL13RA1, SERPINE1, OSMR, CTGF, IGFBP-2, and IRF7) in relation to the trajectory of skin score over time. Subjects expressing high levels of CD14, IL13RA1, SERPINE1, OSMR and CTGF at baseline were more likely to be associated with a progressive trajectory of skin score (Table 3). No association was found between levels of either IRF7 or IGFBP2 gene expressions with skin score trajectories. We further examined the performance of each of these genes as prognostic biomarkers of progressive versus stable/regressive skin disease. Expression of five genes (CD14, IL13RA1, SERPINE1, OSMR and CTGF) was prognostic for the risk of skin progression (Supplementary Table 2). IL13RA1 was performing the best, followed by OSMR and SERPINE1.

Association of Gene expression and skin score change

Expression of several genes, i.e., CD14, IL13RA1, SERPINE1, OSMR, and CTGF were also associated with skin score change over time from baseline. Compared with those in the lowest tertile, patients in the highest tertile of CD14, IL13RA1, SERPINE1, OSMR, and CTGF showed an increased MRSS. In contrast, the highest tertile of IGFBP2 appeared to show an improvement of MRSS over time (Table 4). Similar results were also observed in the validation group.

Discussion

Predicting the trajectory of skin disease in dcSSc patients is currently difficult on the basis of clinical criteria [13]. Analyzing skin gene expression, we show that CD14, SERPINE1, IL13RA1, CTGF, and OSMR mRNA expression are prognostic for the

trajectory of skin disease in active dcSSc patients for one year following the skin biopsy. Thus, increased expression of these genes may serve as better markers than currently available methods for selecting patients with progressive skin disease.

In this study we utilized skin biopsy samples from patients treated with PBO in the Roche faSScinate phase 2 study of tocilizumab in SSc [8]. These samples provided a rare opportunity to examine prognostic biomarkers in a group of active dcSSc patients who were not treated with any immunosuppressive drug. However, the inclusion criteria for this study may have impacted the results. All patients had active dcSSc of ≤5years disease duration since their first non-Raynaud symptom, and a screening MRSS between 15 and 40. In addition, at screening, active progressive disease of <1 year's duration was required as defined by an increase of ≥3 MRSS units, involvement of one new body area with increase in MRSS ≥2 units or two new body areas with increase in MRSS ≥1 unit, other documentation of worsening skin thickening in the previous 6 months, or ≥1 tendon friction rub plus ≥1 laboratory crite rion (C-reactive protein [CRP] ≥10·0 mg/L, erythrocyte sedimentation rate ≥28 mm/h, or platelets ≥330×1000/μL). Enriching for early active disease by these eligibility criteria may have led to enrollment of patients with more progressive disease. We found that the prognostic biomarkers identified using PBO-treated patients enrolled in the faSScinate study also showed trends in R-values that were prognostic in a cohort of patients from Boston University Medical Center (BUMC). The BUMC patient cohort had received treatment with a variety of immunosuppressive medications [12]. Thus, these prognostic biomarkers may have broader prognostic value in other patients with early diffuse SSc. However, it is also possible that these biomarkers may only act as predictors in the preselected faSScinate cohort.

Defining progressive skin disease, as defined by worsening MRSS, is important to enrich patients for clinical trials where separation between the control and active treatment groups over a relative short period of time (i.e. ≤1 year) is desirable. The three trajectory groups indicated that the patients most likely to progress showed high baseline MRSS. We found that only 25% of PBO treated patient showed a progressive trajectory, despite an effort to enrich the patient population for active disease. Similar results were found previously in a larger study in which 192 patients with dcSSc were

grouped using latent linear trajectory [3]. On the other hand, the trajectories we describe here appear, on the surface, to be discrepant with a recent study of European patients [14]. The most apparent possible reason for this difference would be in patient selection for the two studies. The EUSTAR database is an observational study that recruits patients with dcSSc with a broad range of disease durations, whereas faSScinate is a clinical trial of patients with early dcSSc with elevated acute-phase reactants. Thus, the differences in the study population characteristics may explain the difference between these studies and ongoing clinical trials, which have defined inclusion criteria based on the EUSTAR database, will provide further insights on enriching SSc study patients with progressive skin disease.

We show here that macrophage markers, CD14, IL13RA1, MSR1, CD163 and MS4A4A, correlate with progressive skin disease trajectories. Our current findings are in line with previous studies by our group, showing that the macrophage markers, SIGLEC-1 and MRC1 (mannose receptor-1) are increased in lesional SSc skin [15 16]. In addition, we recently showed that changes in skin gene expression of MS4A4A correlates highly with changes in the MRSS, helping define a two-gene pharmacodynamic biomarker [17]. Further, in peripheral blood mononuclear cells, IL13RA1 gene expression correlates highly with pulmonary arterial hypertension in patients with limited cutaneous SSc [18]. Finally, we recently reported that SSc patient treatment with TCZ results in the down-regulation of skin CD14 expression [8]. Together these observations indicate an important function of macrophages in SSc tissue inflammation and fibrosis. As these cells are found surrounding blood vessels, these data suggest that macrophages bridge the fibrotic and vascular features with the pathology seen in SSc skin.

Two of the prognostic biomarkers identified here, SERPINE1 and CTGF, are strongly induced by TGF-β [19 20]. TGF-β has long been suspected as an important mediator of fibrosis in SSc as well as a variety of other fibrotic diseases, including renal, pulmonary, cardiac, and liver fibrosis [21-23]. These two genes were significantly decreased in patients treated with fresolimumab (anti-TGF-β antibody), further supporting the role of TGF-β in pathogenesis of this disease [17].

Finally, we identify OSMR, which forms the Oncostatin (OSM) receptor with the common signaling partner gp130, as a prognostic biomarker. OSM, an IL-6 family cytokine, is produced by a variety of immune cells, including macrophages, neutrophils and activated T cells [24]. It has been implicated in a number of biological processes including the induction of inflammation and the modulation of extracellular matrix (ECM). OSM is upregulated in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and SSc [24] and increased in the serum of dcSSc patients [25].

In conclusion, patients with elevated expression of CD14, CTGF, IL13RA1, OSMR, and SERPINE1 at baseline are more likely to have progressive skin score trajectories. The use of these biomarkers might help to guide decisions about which patients should be considered for aggressive therapies and/or for clinical trials. This observation will be further explored in the ongoing phase 3 focuSSced study of TCZ in SSc patients (ClinicalTrials.gov: NCT02453256).

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Table 1. Patient characteristics

Values are mean±SD, number (%) or as indicated. All patients met the 1980 ACR criteria for systemic sclerosis (SSc). Disease duration was defined by the time since the first non-Raynaud's symptom. (MRSS, modified Rodnan Skin Score).

Characteristic	PBO (N = 38)
Age (Year)	
Mean (SD)	47.2 (13.0)
Median (Range)	49.5 (19-69)
Sex	
Female (n)	78.9% (30)
Male (n)	21.1% (8)
MRSS	
Mean (SD)	25.1 (5.2)
Median (Range)	25 (15-37)
Disease duration (Months)	
Mean (SD)	19.8 (16.8)

Table 2. Comparison of correlations (r) between gene expression and changes of skin score in the discovery group and the validation group

GENE	DISCOVERY GROUP (r)	VALIDATION GROUP (r)
IL13RA1	0.6	0.25
SERPINE1	0.54	0.31
OSMR	0.52	0.27
CTGF	0.45	0.23
CD14	0.59	0.36
IRF7	-0.2	-0.24
IGFBP2	-0.44	-0.32

Table 3. Association of gene expression and trajectory of skin score over follow-up period

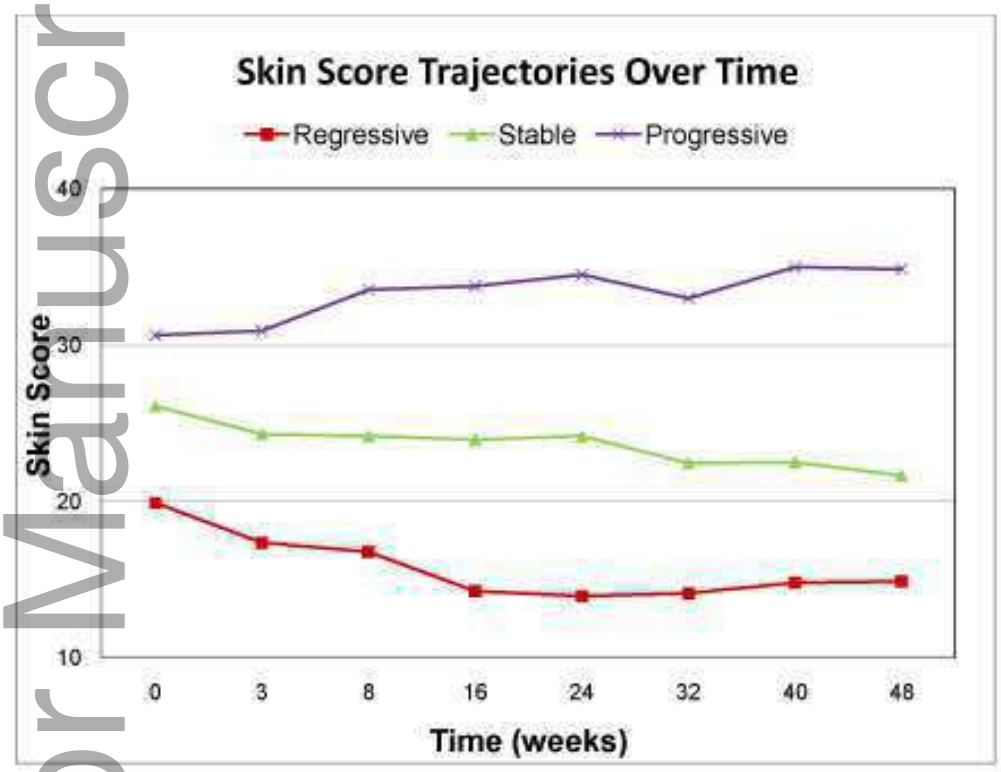
Gene expression	Skin Score Trajectories			P value
	Regressive	Stable	Progressive	
CD14				0.013
Low	5	6	1	
Med	4	7	2	
High	0	5	8	
IL13RA1				0.026
Low	4	7	1	
Med	5	6	2	
High	1	4	8	
SERPINE 1				0.049
Low	5	6	1	
Med	4	6	3	
High	1	4	8	
OSMR				0.058
Low	6	6	0	
Med	2	6	5	
High	2	5	6	
CTGF				0.020
Low	5	5	2	

Med	4	8	1	
High	1	4	8	
IRF7				0.345
Low	5	6	1	
Med	2	6	5	
High	3	5	5	
IGFBP2				0.566
Low	3	4	5	
Med	3	8	2	
High	4	5	4	

Table 4. Association of gene expression and skin score change over the time.

Gene Expression Tertile groups	Mean of skin score change (95% Confidence Intervals)	**P value**
CD14		0.0793
Middle vs lowest	-0.28 (-3.46, 2.90)	0.86
Highest vs lowest	3.48 (0.31, 6.66)	0.03
IL13RA1		0.0532
Middle vs lowest	1.63 (-1.51, 4.77)	0.31
Highest vs lowest	4.08 (1.36, 6.80)	0.003
SERPINE 1		0.0696
Middle vs lowest	-0.74 (-3.99, 2.51)	0.65
Highest vs lowest	3.16 (0.59, 5.72)	0.016
OSMR		0.0184
Middle vs lowest	1.07 (-1.93, 4.07)	0.48
Highest vs lowest	4.08 (1.87, 6.29)	0.0003
CTGF		0.0491

Middle vs lowest	-0.71 (-3.86, 2.44)	0.66
Highest vs lowest	3.32 (0.87, 5.76)	0.008
IRF7		0.4539
Middle vs lowest	0.90 (-1.53, 3.32)	0.44
Highest vs lowest	-1.23 (-4.39, 1.93)	0.47
IGFBP2		0.1241
Middle vs lowest	0.37 (-1.94, 2.69)	0.75
Highest vs lowest	-3.10 (-6.29, 0.10)	0.058



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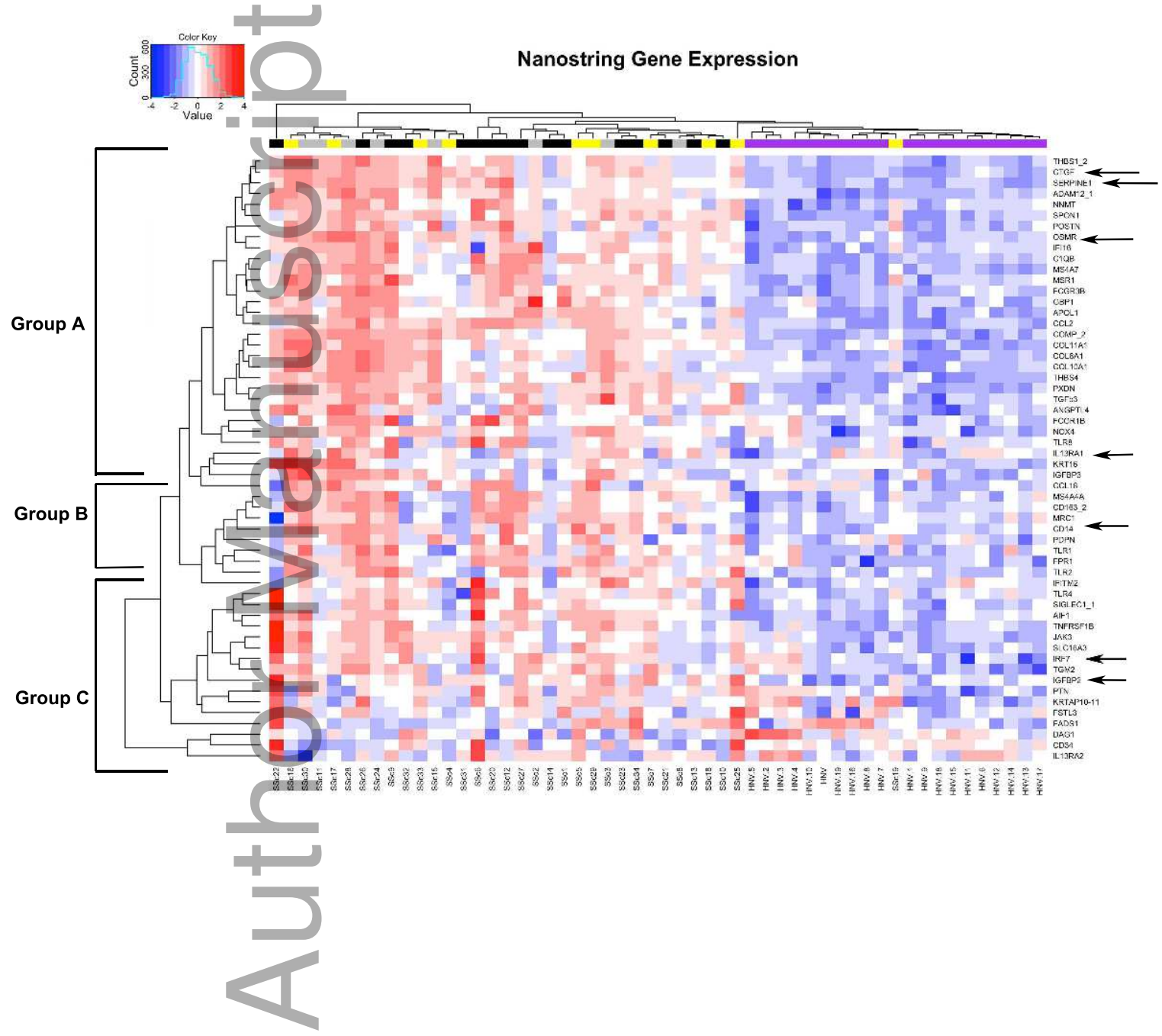

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