UBASH3A and TIGIT Genes Expression Levels in Systemic Sclerosis

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Abstract

Systemic sclerosis (Ssc) is an autoimmune disorder marked by excessive fibrosis, microvascular stenosis, and systemic clinical manifestations. An autoimmune process is believed to induce T-cell activation, mainly CD 4 T helper cells, and enhance production of proinflammatory and profibrotic cytokines such as IL 4 and IL 13. These cytokines contribute to vasculopathy and excessive collagen synthesis. UBASH3a and TIGIT are coinhibitory receptors expressed on T cell to suppress T cell activation. Our study aimed to explore UBASH3A and TIGIT mRNA expression levels in systemic sclerosis (Ssc) patients compared to healthy controls. We detected the mRNA levels via real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) in total RNA, isolated from the peripheral blood mononuclear cells (PBMCs) of 30 Ssc patients and 30 age and sex matched healthy controls with RNA extraction kits. The expression level of UBASH3A and TIGIT mRNA was significantly high in PBMCs from Ssc patients in comparison with healthy subjects.

Keywords: *UBASH3A, TIGIT, T cell, Systemic Sclerosis*

1. Introduction

Systemic sclerosis (Ssc) is an autoimmune disease, which is characterized by skin tightness and systemic

manifestations. Environmental and genetic factors is likely to be involved in Ssc etiology (1). Pathogenesis includes vascular changes, evidence of autoimmunity with distinct autoantibodies and activation of innate and adaptive immunity and skin and organ fibrosis that cause irreversible organ damage which contributes to high morbidity and mortality (2). Cases with significant pulmonary or cardiac involvement have 3-year survival rate of 47-56% (3).

In Ssc, T cells are proven to have a significant role. They infiltrate tissues very early in the disease course. In-situ hybridization studies revealed that infiltrating T cells and macrophages are neighboring to myofibroblasts, explaining the link between immune cells and fibrosis (4). An antigen-specific immune response is suggested by the oligoclonal repertoire displayed on T lymphocytes isolated from the blood or fibrotic skin of Ssc patients (5).

Moreover, a breakdown in self-tolerance is believed to induce production of proinflammatory and profibrotic cytokines, which participate in vasculopathy and excessive collagen synthesis (6). Patients with active Ssc demonstrated high levels of CD4 and CD8 T cells in blood and skin which secrete profibrotic type-2 cytokines such as interleukin (IL)-4, IL-13 and IL-17, driving inflammatory mechanisms involving fibroblasts, endothelial and epithelial cells (7). A deficient or altered function of T regulatory cells (Tregs) may also_induce altered immune response and fibrosis in Ssc (8).

UBASH3a is a type of protein tyrosine phosphatase family that is involved in tyrosine phosphorylation regulation within T cells and the negative regulation of T-cell signaling (9). UBASH3A deficiency eases T-cell responses to T-cell receptor (TCR)/CD3 complex stimulation, thus aggravating T-cell-dependent inflammation (10).

It enhance T cells apoptosis via binding to the apoptosis-inducing protein apoptosis-inducing factor (AIF), a key factor of caspase-independent apoptosis (11).

Genetic polymorphisms and altered expression levels of UBASH3A were associated with multiple autoimmune diseases (Ads), such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) , and Type 1 Diabetes (12).

TIGIT is part of the poliovirus receptor (PVR). Grogan, et al. in 2009 described it as an inhibitory receptor that inhibits T cell via TCR downregulation, and stimulates the production of the immunosuppressive molecule, FGL2. Also, Ligation of PVR with TIGIT stimulates IL-10 production from regulatory T cells (14).

So, a better understanding of the immunological process including the role of coinhibitory receptors (Co-IRs) in Ssc will guide novel therapeutic approaches.

2. Patients & Methods

2.1. Patients

This hospital-based cross-sectional case-control study included 30 Ssc patients and 30 healthy volunteers. Patients were recruited from inpatient and outpatient settings of the Rheumatology, Rehabilitation, and Physical Medicine department in tertiary care hospitals in the period between March 2023 and October 2023. Ethical approval was

obtained from the local faculty of medicine ethical committee (No O4-2023-300172). Study subjects were provided with an adequate explanation of the protocol and alternatives. We obtained informed consent from all study subjects. Patients were included in the study if they were older than 18 years old, and had established Ssc diagnosis according to the ACR-EULAR classification criteria of Ssc (15). Patients were excluded if they had evidence of other rheumatological disease or malignancy.

Complete history taking, clinical assessment, and routine laboratory investigations were done. Clinical assessment included:

- Modified rodnan skin score: it is used to evaluate skin thickness graded by clinical palpation from 0 to 3 (0=normal skin; 1=mild thickness; 2=moderate thickness; 3=severe thickness). Seventeen surface anatomic areas are examined; face, anterior chest, abdomen, (right and left) fingers, forearms, upper arms, thighs, lower legs, dorsum of hands and feet. Separate values are summed to get the total skin score. (16, 17)
- Valentini Disease Activity Index: it is 10 items questionnaire. Each item has a constant numerical value from 0.5 - 2.0. The total score ranges from 0 to 10. The items are Modified Rodnan skin score > 14, scleredema, changes in skin stiffness during one month, digital necrosis, vascular symptoms changes during one month, arthritis, diffusion lung capacity < 80%, cardiopulmonary symptoms changes, erythrocyte sedimentation rate >30mm/1.hour and hypocomplementemia (18).

2.2. Materials & Methods

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn EDTA blood by Ficoll-Paque gradient centrifugation according to the manufacturer's protocol. The PBMCs were stored at −80°C.

The total RNA was extracted from the blood samples of all the groups using RNA extraction kit. The RNA concentration was assessed using nanodrop spectrophotometer. The RNA was reverse-transcribed into cDNA. RTqPCR was in a 20 μL volume that contains 10 μL of SYBR Premix Ex Taq II, 0.1 μg of cDNA template, 1 μL of forward primers (final concentration 0.4 μmol/L), and 1 μL of reverse primers (final concentration 0.4 μmol/L). Relative expressions were normalized to β-actin for endogenous control. ΔCt will was recorded automatically and relative expression levels of coinhibitory receptors was calculated by the $2^{-\Delta\Delta Ct}$ method. The used primer sequences are listed below.

- TIGIT
- F: 5′TCTGCATCTATCACACCTACCC3′
- R: 5′CCACCACGATGACTGCTGT3′
- UBASH3A
- F: 5′-GGACTTG-CACGACTAA-3′
- R: 5′-CCGTACGTCAATTGAC-3′
- β-ACTIN
- F: 5′AGAGCTACGAGCTGCCTGAC3′

- R: 5′AGCACTGTGTTGGCGTACAG 3′

The fold changes (FCs) of the target mRNAs were normalized to β-ACTIN, and then, the FCs of each mRNA were calculated based on the ratio between the patient groups and healthy controls as indicated. The experiment was repeated three times in triplicate to confirm the results; the threshold cycle value ($2^{-\Delta CT}$) was used for statistical analysis and the results are presented as fold changes (FCs).

2.3. **Statistical analysis**

Data analysis was undertaken using SPSS version 20. Categorical data were presented in the form of frequencies and percentages. Numerical data is checked for normality by Shapiro- walk test and presented by mean and standard deviation or median and range.

The Mann-Whitney U test was used to compare the median difference of the FCs of UBASH3A and the FCs of TIGIT between patients and controls, spearman's correlation was used to identify the correlation between FCs of UBASH3A, FCs of TIGIT and different variables.

3. Results

Table (1): Sociodemographic data of Ssc patients

Variables	n=30	%
Age (Years)		
Mean ±SD (Range)	41.77±9.73 (18.0-60.0)	
Gender		
▪ Male	4	13.3%
▪ Female	26	86.7%
Marital status		
▪ Married	23	76.7%
▪ Single/widow/divorced	7	13.3%
Smoking		
▪ Smoker	1	3.3%
▪ Passive smoker	17	56.7%
▪ Non-smoker	12	40.0%
Family history of Rheumatological disease	6	20.0%
Anthropometric measures		
▪ Height (cm)	162.57±5.60	
▪ Weight (kg)	65.13±14.05	

▪ BMI	24.53±4.58

Data expressed as mean± SD (range) or frequency (%)

Regarding demographic characteristics, the mean age of patients with Ssc was 41.77±9.73 and ranged from 18 to 60 years, 86.7% were females and 76.7% were married. 56.7% were passive smokers and 40 % non-smoker. The mean BMI was 24.53±4.58 (Table 1). No statistically significant difference between patients with Ssc and controls regarding age, sex, and other demographic data.

Table (2): clinical manifestation of the patients with Ssc

Variables	n=30	(%)
Disease Duration in years: Median (range)	7.0 (1.5-21.0)	
Age of disease onset: Median (range)	35.50 (7-55)	
Musculoskeletal manifestation		
▪ Arthralgia	17	56.7%
▪ Arthritis	2	6.7%
▪ Myalgia	2	6.7%
Vascular and mucocutaneous manifestations		
▪ Raynaud's Phenomenon	30	100.0%
▪ Digital scar	27	90.0%
▪ Telangiectasia	10	33.3%
▪ Salt and pepper	9	30.0%
▪ Digital ulcer	7	23.3%
▪ Dry eye	10	33.3%
▪ Dry mouth	8	26.7%

Chest and Cardiovascular manifestations		
▪ IPF	22	73.3%
▪ PASP: Mean ± SD (range)	28.30±8.69 (12-57)	
Total Modified rodnan: Mean ± SD (range)	17.83±5.86 (9-34)	
Total valentini activity index: Mean ± SD (range)	3.13±1.56 (0.5-7.0	
Disease subset		
▪ Limited	21	70.0%
▪ Diffuse	9	30.0%

Data expressed as mean± SD/ median(range) or frequency (%)

PASP (pulmonary artery systolic pressure) Regarding clinical manifestations of patients with Ssc, median duration of disease was 7 years and ranged from 1.5 to 21.0 years, median age of onset of disease was 35.50 years and ranged from 7.0 to 55.0 years.

The most common musculoskeletal manifestation was arthralgia (56.7%). Regarding vascular and mucocutaneous manifestations, all patients complained of Raynaud's Phenomenon, 90.0% from digital scar, 33.3% from Telangiectasia, 30.0% from salt and pepper, and 23.3% from digital ulcer. Regarding chest and cardiovascular manifestations, 73.3% of patients had interstitial pulmonary fibrosis (IPF), and mean PASP was 28.30±8.69. About one third of patients complained of dry eye and 26.7% had dry mouth. The mean total modified rodnan was 17.83±5.86 and the mean total valentini activity index was 3.13±1.56. 70.0% of patients have the limited type of disease and 30.0% have the diffuse type (Table 2).

Table (3): laboratory investigations among Ssc patients

Parameter	N=30
CBC	
▪ WBCs (x 10^6/L): Mean ± SD	5.89±1.89
▪ Neutrophils%: Mean ± SD	57.84±12.73
▪ Neutrophils absolute (x 10^6/L): Mean ± SD	3.49±1.74
▪ Lymphocytes %: Mean ± SD	29.79±11.05
▪ Lymphocytes absolute (x 10^6/L): Mean ± SD	1.66±0.72
▪ NLR: median (range)	1.6 (0.68-10.37)
▪ RBCs (x 10^9/L): Mean ± SD	4.35±0.61
▪ Hgb (g/dl): Mean ± SD	12.08±1.54
▪ Platelets (x 10^6/L): Mean ± SD	272.20±85.97
Inflammatory markers	
▪ ESR (mm/hr): median (range)	35.0 (3.0-107)
▪ CRP (mg/l): median (range)	4.60 (0-22.0)
▪ C 3 (mg/dl)	124.89±25.17

▪ C 4 (mg/dl)	25.73±7.44
ANA	
▪ Positive	28 (93.3%)
▪ Negative	2 (6.7%)

Data expressed as mean ±SD or median (range)

Table (3) shows laboratory investigations among Ssc patients, mean WBCs count was 5.89±1.89 x 10^6/L, mean Neutrophils% was 57.84±12.73, mean Neutrophils absolute was 3.49±1.74 x 10^6/L, mean lymphocytes % was 29.79±11.05, mean lymphocytes absolute was 1.66±0.72 x 10^6/L, median NLR was 1.6 and ranged from 0.68 to 10.37, mean RBCs was 4.35±0.61 x 10^9/L, mean Hgb was 12.08±1.54 g/dl, mean platelet count was 272.20±85.97 x 10^6/L. The median ESR level was 32.0 and ranged from 3.0 to 107, Median CRP level was 4.60 and ranged from 0.0 to 22.0. 93.3% were positive for ANA.

Table (4): Gene expression among patients with Ssc patients and controls

Variables	Ssc (n=30)	Controls (n=30)	P-Value*
FCs of UBASH3A			
▪ Median (range)	2.31 (0.53-19.55)	1.36 (0.01-7.25)	0.011
FCs of TIGIT			
▪ Median (range)	3.00 (2.40-26.00)	1.45 (0.07-2.70)	<0.001

Data expressed as median (range)

*Mann Whitney U test

Figure (1): Boxplot for the distribution of FCs of UBASH3A among Ssc patients and controls

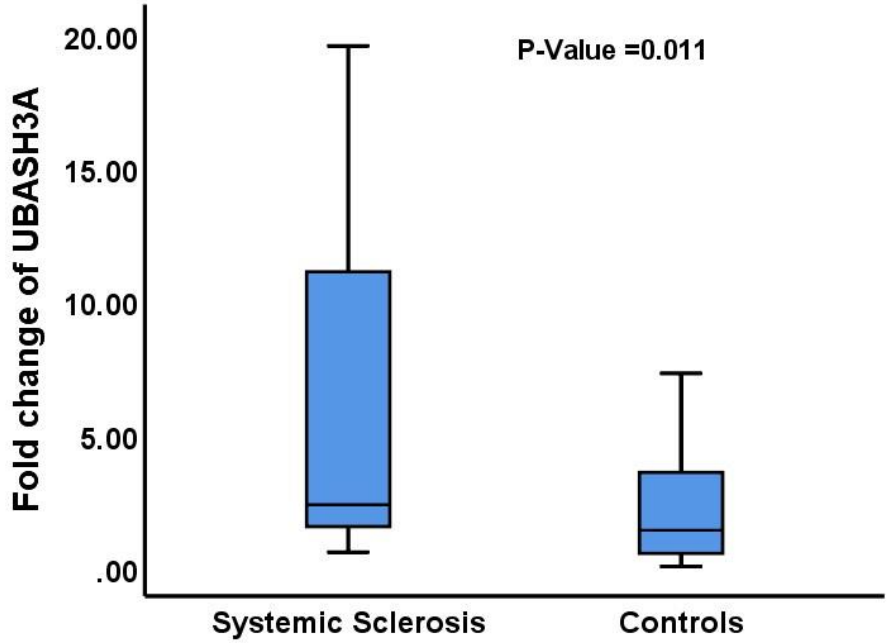

Table (4) and figure (1) show a statistically significantly higher median expression of FCs of UBASH3A among patients with Ssc compared with control.

Figure (2): Boxplot for the distribution of FCs of TIGIT among Ssc patients and controls

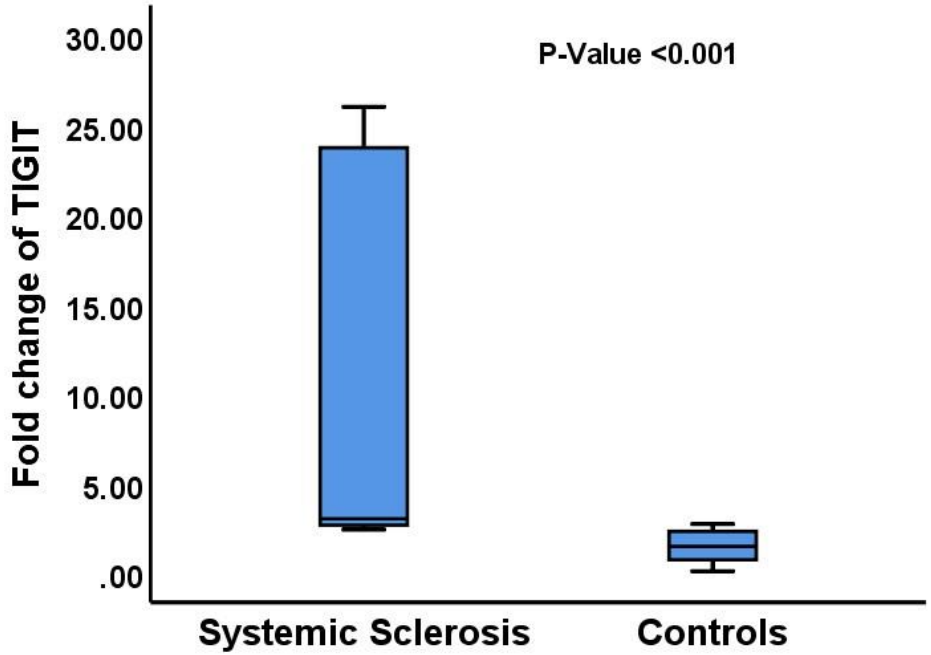
P-Value <0.001
Fold change of TIGIT
30.00
25.00
20.00
15.00
10.00
5.00
.00
Systemic Sclerosis
Controls

Table (4) and figure (2) show a statistically significantly higher median expression of FCs of UBASH3A among patients with Ssc compared with control.

Table (5): Correlation between FCs of UBASH3A and FCs of TIGIT and other variables among patients with Ssc

Variables	FCs of UBASH3A		FCs of TIGIT	
	R	p-value*	R	p-value*
FCs of TIGIT			0.596	0.001
Age	0.214	0.256	0.074	0.698
Disease Duration in years	0.009	0.962	0.051	0.789
Age of disease onset	0.207	0.273	0.074	0.696
Total Modified rodnan	0.191	0.312	0.306	0.100
Total valentini activity index	-0.152	0.423	0.166	0.381
PASP	0.262	0.162	0.030	0.876
Investigation				

▪ WBC (x 10^6/L)	0.096	0.614	0.075	0.692
▪ Neutrophils %	0.142	0.453	0.110	0.562
▪ Neutrophils absolute (x 10^6/L)	0.145	0.443	0.157	0.408
▪ Lymphocytes %	-0.088	0.644	-0.074	0.697
▪ Lymphocytes absolute (x 10^6/L)	0.017	0.928	0.012	0.949
▪ NLR	0.105	0.579	0.094	0.622
▪ RBCs (x 10^9/L) _	0.057	0.767	-0.116	0.542
▪ Hgb (g/dl)	0.021	0.912	-0.131	0.490
▪ Platelets (x 10^6/L)	0.056	0.771	0.082	0.667
▪ ESR (mm/hr)	0.052	0.783	0.168	0.375
▪ CRP (mg/l)	0.178	0.346	0.415	0.022

*Spearman correlation r (correlation coefficient)

Table (5) revealed a statistically significant

positive moderate correlation between the expression of the FCs of TIGIT and the FCs of UBASH3A among patients with Ssc (r=0.596, p-value =0.001). Moreover, there was a statistically significant positive moderate correlation with CRP level (r=0.415, p-value =0.022).

No statistically significant correlation between the expression of FCs of TIGIT or FCs of UBASH3A with; age, disease duration, age of disease onset, total modified rodnan, total valentini activity index, PASP, and other laboratory investigations.

Table (6): association between FCs of UBASH3A and FCs of TIGIT and types of Ssc

Variables	FCs of UBASH3A	FCs of TIGIT
Type of Disease		
▪ Limited	1.70 (0.90-19.55)	2.79 (2.40-26.00)
▪ Diffuse	2.53 (0.53-19.53)	24.00 (2.60-25.00)
P-Value*	0.230	0.007

Data expressed as median (range)

*Mann Whitney U test

Table (6) revealed statistically significantly higher median expression of the FCs of TIGIT among patients with diffuse Ssc compared to limited type (24.0 compared to 2.79 respectively), p-value =0.007. However, there was no statistically significant difference between limited and diffuse Ssc regarding the expression of FCs of UBASH3A.

4. Discussion

TGIT and UBASH3A, as Co-IRs, show an important role in limiting unwarranted T cell activation and in maintaining immune tolerance, thus they assume a significant role in unraveling Ssc pathogenesis (19).

This study analyzes the expression levels of two Co-IRs TIGIT, and UBASH3A in SSc patients comparable to healthy subjects and correlates these expression levels with the clinical and laboratory data of the studied subjects.

Statistically significant higher median expression of FCs of TIGIT among patients with Ssc compared to controls was detected. Also, a statistically significant higher median expression of FCs of TIGIT among patients with diffuse Ssc compared to limited type was detected. These results match with those reported by Michelle, et al., that TIGIT as Co-IRs were highly expressed mainly in T cells and NK cells in Ssc patients in comparison to healthy subjects. Also, CD4+ T cells and Treg cells from Ssc patients had an increased level of PD-1+TIGIT+ double-positive cells compared to those from healthy controls. Exhausted T cells from overwhelming autoimmune processes are characterized by high expression of multiple Co-IRs on the cell surface (20).

Increased TIGIT expressing CD4+ T cells has been reported in other ADs. SLE patients showed significantly higher expression of TIGIT on CD4+ T cells, specifically in those with high levels of anti-double stranded DNA antibody, anti-Sm, and urinary protein (21). Another study detected upregulation of TIGIT on T cells in RA patients (22). CD4+ TIGIT+ T cells levels in synovial fluid are negatively correlated to the disease activity in RA patients (23).

In atopic dermatitis, TIGIT expressing CD4+ T cells has increased compared with healthy subjects, but the four most severe atopic dermatitis cases showed a significant reduction in TIGIT+ CD4+ T cells numbers [38]. This study suggests that disease severity may be attributed to a reduction in TIGIT+ CD4+ T cells due to immune exhausion (24).

TIGIT+ Tregs function to control autoimmune thyroiditis which is proved by an association between the expression of an inhibitory receptor Fc Receptor-Like 3 (FCRL3) and TIGIT in various subsets of autoimmune thyroiditis [40].

In type 1 diabetes, TIGIT+ Tregs have also been shown to be increased. TIGIT+CD226- Tregs are suppressive, compared to TIGIT-CD226+ T cells with a reduced suppressive function, effector cytokine and IL-10 production (25).

On the other side, multiple sclerosis (MS) patients showed lower TIGIT-expressing CD4 cells (26). However in another study, TIGIT signaling pathway was found to be active and TIGIT stimulation of PBMCs from MS patients reduces Th1 differentiation (26). These studies suggest that TIGIT-expressing Tregs are involved in the suppression of MS. The reduced level of TIGIT+ Tregs may contribute to the disease in active MS patients (27).

A statistically significant positive moderate correlation between TIGIT and CRP levels has been reported. This result matches with those that reported that the levels of TIGIT+ on CD4+ and CD8+ T cells are positively correlated with the level of CRP in sepsis patients (28) and RA patients (22). The relationship between these predictors of

inflammation and frequency of TIGIT+ CD4+ T cells may indicate an increased intensity of chronic inflammation (22).

A growing evidence has explained the link between UBASH3A gene and several ADs (29). This study is the first study that detects UBASH3A mRNA expression levels in Ssc. However, there was no statistically significant difference between limited and diffuse Ssc regarding the expression of FCs of UBASH3A. In contrast to previous studies conducted on other ADs, our results showed that statistically significant higher expression of UBASH3A among patients with Ssc compared to controls. That may be explained by the increased expression of CO-IRs on exhausted T cell surfaces (20). UBASH3A mRNA expression levels are remarkably decreased in SLE patients compared to healthy subjects. Moreover, there was a negative correlation between UBASH3A mRNA expression levels and disease activity in SLE patients.

Also, the expression of UBASH3A gene was extremely low in fibroblast-like synoviocytes in patients with RA (30). Decreased UBASH3A mRNA expression in SLE and RA indicated that UBASH3A might have acted as a "protective" factor in these diseases.

Genetic variants in *UBASH3A* have been associated with at least five distinct ADs (31). Previous studies also showed that its role in T cells supports their contribution in the maintenance of immunological hemostasis, which protects against autoimmune and chronic inflammatory conditions. The major effect of UBASH3A on T cells may be linked to its role in AIF-mediated apoptosis. UBASH3A deletion may act by increasing the persistence of activated T cells (32).

A statistically significant positive moderate correlation between expression of FCs of TIGIT and FCs of UBASH3A among patients with Ssc. Fleury and his colleagues demonstrated that the upregulated expression of CoIRs, that are involved with T cell exhaustion processes, is attributed to peripheral blood Tregs (20). They observed elevated expression of PD-1 and TIGIT in other T-cell subsets and natural killer cells which support a cell-specific altered expression pattern of Co-IRs in Ssc immune cells, rather than a broad up-regulation of these receptors.

The previous observations indicate that in some Ads, the expression of Co-IRs such as TIGIT+ T cell population may be increased to control disease, however in other cases, this may be an indicator that the disease has progressed into a more severe condition (33).

5. Conclusion and Recommendation

In this study, we detected increased expression of TIGIT and UBASH3A3 in SSc patients which indicates that those patients express CO-IRs signatures reminiscent of immune cell exhaustion so they may be candidate for targeted therapy. It is suggested that further functional analysis on their roles in the development and progression of Ssc, their mechanisms involved in the regulation of Tcell activation and suppression, and their association with the development of autoimmune disorders should be conducted.

6. Limitations to the study

The small sample size, and lack of functional characterization of the studied genes were considered to be limitations of the study, and it should not be ignored while interpreting the results. However, the present study has strengths regarding the well-matched cases and control population.

7. List of abbreviations

Ads	Autoimmune diseases
Co-IRs	Co-inhibitory receptors
FCs	Fold Changes
IL	Interleukin
MS	Multiple sclerosis
PASP	Pulmonary artery systolic pressure
PVR	Poliovirus receptor
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
Ssc	Systemic sclerosis
TCR	T-cell receptor

8. AUTHOR CONTRIBUTIONS

All authors participated in drafting the article and reviewing it critically for important intellectual aspects, and all authors approved the final version to be published. Dr. Aml Adel Rayan Mohammed and Dr. Fatma Mohammed Helbawi had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Dr Fatma Mahmoud abdelraheem Mohamed[4]

9. Avilability of data and materials

The used and/or analyzed datasets during the current study are avilable from the corresponding author upon reasonable request.

10. Ethics

This study was conducted following the Declaration of Helsinki, and it was approved by the Ethical committee, faculty of Medicine, Assuit University with registry number 04-2023-300172

11. Financial support None declared.

12. Conflicts of interest

The authors declare no conflict of interest.

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