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INTERLEUKIN-1β AND *TUMOR NECROSIS FACTOR-α* GENE POLYMORPHISMS IN SYSTEMIC SCLEROSIS

Hakami M.A[1], Alotaibi B.S[1], Alkhalil S.S[1], Das S[2], Nasreen N[3], Jeraiby M.A[4], Jawed A[5], Lohani M[5], Dar S.A[5*]

[*]**Corresponding Author:** Sajad Ahmad Dar, Department of Nursing, College of Nursing and Health Sciences, Jazan University, Jazan – 45142, Saudi Arabia; Email: sdar@jazanu.edu.sa

ABSTRACT

The complex cytokine network plays an important role in disease susceptibility and development, therefore single-nucleotide polymorphisms (SNPs) in or near cytokine genes may be relevant to development of systemic sclerosis (SSc). We in this study investigated 22 SNPs in 13 cytokine genes of SSc patients, and their association with disease susceptibility. Twenty-three clinically diagnosed SSc patients were enrolled for this purpose along with 80 healthy volunteers for comparisons. Aseptically collected 2ml of peripheral venous blood from each subject was processed for DNA extraction. Cytokine genotyping was carried out using the extracted genomic DNA by PCR employing sequence-specific primers and data was analyzed for any association with SSc susceptibility. Variations in allele, genotype, or haplotype distribution between patients and healthy volunteers were observed for the following SNPs: *IL-1β* –511 C/T (rs16944) and +3962 T/C (rs1143634); *IL-4Rα* +1902 G/A (rs1801275); *IL-12* –1188 C/A (rs3212227); *TGF-β1* codon 25 G/C (rs1800471); *TNF-α*–308 G/A (rs1800629) and –238 G/A (rs361525); *IL-4* –1098 T/G (rs2243248) and –590 T/C (rs2243250); *IL-6* –174 G/C (rs1800795) and nt565 G/A (rs1800797); and *IL-10* –1082 G/A (rs1800896), –819 C/T (rs1800871) and –592 C/A (rs1800872). However, only the SNPs in *IL-1β* –511 and +3962, and *TNF-α* –308 and –238 were found to be significantly associated with SSc susceptibility. Our findings suggest that *IL-1β* and *TNF-α* gene SNPs may play a role in development of SSc, although large observational and experimental studies are needed to substantiate these findings.

Keywords: Autoimmunity; Cytokines; Disease development; Genotyping; Predisposition

INTRODUCTION

Systemic sclerosis (SSc) is a generalized disorder of small arteries, microvessels, and connective tissue. It is a disease of unknown origin, with the highest incidence occurring between 45 to 55 years of age [1]; the frequency is three to eight times higher in females [2]. Several studies have demonstrated that the extent of skin involvement directly correlates with internal organ involvement and prognosis in SSc patients [3, 4]. Manifestations associated with SSc have been found to negatively impact the quality of life in affected individuals [5].

Long-term occupational exposure to environmental toxins is a common finding in SSc patients [6]. However, the effect of these environmental toxins on immune system of these genetically susceptible patients is unclear. Recent studies have raised the possibility that both genetic and environmental factors act synergistically at several stages of autoimmunity pathogenesis. These studies predict that individuals susceptible to spontaneous autoimmunity should be more susceptible following xenobiotic exposure by virtue of the presence of predisposing background genes [7]. Studies have shown that genetic predisposition plays an important role in susceptibility and the development

[1] Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Al- Quwayiyah, Shaqra University - 19254, Riyadh, Saudi Arabia.

[2] Department of Microbiology, University College of Medical Sciences (University of Delhi) & GTB Hospital, Delhi - 110095, India.

[3] Department of Oral Pathology and Microbiology, Divya Jyoti College of Dental Sciences and Research, Modinagar - 201204, Ghaziabad, U.P., India.

[4] Department of Basic Medical Science (Medical Biochemistry), Faculty of Medicine, Jazan University, Jazan - 45142, Saudi Arabia.

[5] Department of Nursing, College of Nursing and Health Sciences, Jazan University, Jazan – 45142, Saudi Arabia.

of autoimmune diseases. This is likely due to functional polymorphisms within multiple genes, each of which, by modulating corresponding protein expression, influences disease susceptibility.

Cytokines may promote the deposition of collagen and fibrosis [8] and many studies have focused on the role of these mediators in SSc, enlisting alterations in their concentrations [9, 10] or in the balance between Th1 and Th2 cytokine levels [11]. Because cytokine production is regulated at the genetic level [12, 13], it has been hypothesized that single-nucleotide polymorphisms (SNPs) in or near cytokine genes may be relevant to the development of SSc.

Numerous studies examining patients with SSc from diverse ancestral backgrounds have identified SNPs in various cytokine genes. Among these, the *IL-1* cytokine family has emerged as particularly significant in the pathogenesis of SSc. There is compelling evidence linking polymorphisms within the *IL-1* family to the disease, as summarized by Xu et al. [14]. Specific gene variants in *IL-1α* [15], *IL-1β* and *IL-2* [16,17], as well as *IL-10* [18,19], have been observed across different populations. However, some associations reported in individual studies [19] have not been consistently replicated in others [17], highlighting the complexity and variability of these genetic links.

These and several other contradictions motivated us to investigate the commonly studied cytokine gene SNPs among our SSc patients, and compare our findings with those previously reported. In this study we evaluated the presence of 22 SNPs in 13 cytokine genes in SSc patients and attempted to associate the significant SNPs with SSc disease susceptibility in our population.

MATERIALS AND METHODS

Samples

Patients clinically diagnosed with SSc, confirmed through established laboratory investigations and meeting the American College of Rheumatology (ACR) criteria [20], were consecutively enrolled at a tertiary care hospital in North India. The rarity of SSc, its genetic heterogeneity, and strict diagnostic criteria present significant challenges in participant recruitment for SNP studies. Additionally, specific disease subtypes, comorbidities, drug exclusions, geographic barriers, and ethical concerns further limit the eligible patient pool.

Our study involved 23 SSc patients and 80 age-matched healthy volunteers of Asian ethnicity. Patients and healthy volunteers were unrelated, and the latter had no clinical history of skin disease, minimizing potential confounders and ensuring clear group distinction. Peripheral venous blood (2ml) was collected aseptically from each patient and healthy volunteers into EDTA vacutainer tubes and used for DNA extraction. The study was approved by the Institutional Ethical Committee-Human Research, and written informed consent was obtained from each patient and healthy volunteer before enrollment in the study.

Genomic DNA Extraction from Blood

Genomic DNA was extracted from blood samples of 23 SSc patients and 80 healthy controls for cytokine genotyping of 22 SNPs in 13 cytokine genes using PCR with sequence-specific primers. DNA extraction was performed using the HiPurA™ blood genomic DNA extraction kit (HiMedia Laboratories) as per the manufacturer's protocol. Briefly, 200µl of blood was mixed with 20µl Proteinase K solution, vortexed, then treated with 20µl RNase A solution. After incubation, 200µl of lysis buffer (C1) was added, followed by a 10-minute incubation at 55°C. Ethanol (200µl) was added, and the lysate was transferred to a spin column for centrifugation. The column was washed with prewash and wash buffers, then eluted with 100µl elution buffer after a 5-minute incubation. DNA was stored at -20°C for PCR analysis.

Cytokine Genotyping by PCR

Cytokine genotyping was carried out from genomic DNA by PCR with sequence-specific primers using commercially available Cytokine Genotyping Kit (Invitrogen Corporation, USA). Twenty two SNPs (*IL-1α* –889 T/C; *IL-1β* –511 C/T and +3962 T/C; *IL-1R* pst1 1970 C/T; *IL-1RA* mspa1 11100 T/C; *IL-4Rα* +1902 G/A; *IL-12* –1188 C/A; *IFN-γ* +874 A/T; *TGF-β1* codon 10 T/C and codon 25 G/C; *TNF-α* –308 G/A and –238 G/A; *IL-2* –330 T/G and +166 G/T; *IL-4* –1098 T/G, –590 T/C, and –33 T/C; *IL-6* –174 G/C and nt565 G/A; *IL-10* –1082 G/A, –819 C/T, and –592 C/A) in thirteen cytokine genes were assessed in all the patients and healthy volunteers using the kit according to the included instructions.

For 48 reactions/wells for each sample, 140µl of PCR buffer was mixed with 3.3µl of Taq DNA polymerase, 329µl of water and 50µl of 75-125ng/µl concentrated DNA template. The reaction mixture (10µl) was dispensed into each well and the following thermal cycler profile was used for amplification. Step 1 was denaturation for 2 minutes at 94°C; Step 2 comprised 10 cycles of 94°C for 15 seconds and 65°C for 60 seconds with no separate extension step; Step 3 (20 cycles) consisted of 94°C for 15 seconds, 61°C for 50 seconds, and 72°C for 30 seconds. The profile was set on hold at 4°C.

The PCR products were loaded onto a 2 percent agarose gel in a specific order for electrophoresis and run at 150 volts for 20-25 minutes for separating the DNA. After electrophoresis, the ethidium bromide stained gel was photographed and interpreted for specific amplification patterns using the worksheet provided with the kit.

Presence of a control band in each lane was ascertained. Wells identifying the *IL-2*, *IL-4*, *IL-6*, and *IL- 10* cytokines contained an 89 bp fragment of the β-globin gene as an internal control. Wells identifying the *IL-1α*, *IL-1β*, *IL-1R*, *IL-1Rα*, *IL-4Rα*, *IL-12*, *IFN-γ*, *TGF-β*, and *TNF-α* cytokines contained a 440 bp fragment of the human C-reactive protein gene as an internal control.

Statistical Analysis

Two-sided Fisher's exact test was used to compare allele, genotype and haplotype frequencies between patients and controls. The threshold for significance was $p<0.05$, and the relative risks associated with rare alleles, genotypes and haplotypes were estimated as odds ratios (ORs) with 95% confidence intervals (CIs). The deviation from Hardy-Weinberg equilibrium (HWE) was determined using a goodness-of-fit Chi-square test to compare the observed genotype frequencies with the expected frequencies among the patients and healthy volunteers. The polymorphisms were excluded if they deviated from HWE. All statistical analyses were performed by SPSS 16.0 (SPSS Inc).

RESULTS

Patients and controls

A total of 23 patients with SSc (4 males, 19 females; mean age 35.5 years) and 80 healthy volunteers (32 males, 48 females; mean age 36 years) were analyzed for 22 SNPs in 13 cytokine genes using cytokine genotyping with sequence-specific primers. The duration of SSc disease ranged from 2 months to 14 years. Common presentations included Raynaud phenomenon, skin sclerosis, and pigmentation, along with finger contractures, digital ulcers, dyspnea, restricted mouth opening, joint issues, and dysphagia. The Rodnan skin score ranged from 9 to 51. The higher proportion of females in the SSc patient group (82.6%) compared to the control group (60%) reflects the well-established female predominance in systemic sclerosis (SSc), with the disease being more prevalent in women. The higher number of healthy volunteers was driven by the rarity of SSc, its strict diagnostic criteria, and the challenges in recruitment, while healthy volunteers are more readily available. This larger control group ensures a robust comparison, minimizes biases, and enhances the study's ability to detect genetic associations, especially given SSc's genetic heterogeneity and clinical variability. All SNPs, except the *IL-12* -1188 C/A ($p<0.05$ for patients and $p<0.01$ for controls), were in Hardy-Weinberg equilibrium ($p>0.05$) for both groups.

Significance of Cytokine Gene Polymorphisms

Distribution of allelic or genotypic frequencies of *IL-1α* –889 T/C (rs1800587); *IL-1RI* pst11970 C/T (rs2234650); *IL-1RA* mspaI11100 T/C (rs315952); *IFN-γ* +874 A/T (rs2430561); *TGF-β1* codon 10 T/C (rs1982073); *IL-2* –330 T/G (rs2069762) and +166 G/T (rs2069763); and *IL-4* –33 T/C (rs2070874) cytokine gene polymorphisms was similar in patients and controls. No statistically significant associations of these SNPs with the disease could be found (data not shown). However, variations in allele, genotype or haplotype distribution were observed in *IL- 1β* –511 C/T (rs16944) and +3962 T/C (rs1143634); *IL-4Rα* +1902 G/A (rs1801275); *IL-12* –1188 C/A (rs3212227); *TGF-β1* codon 25 G/C (rs1800471); *TNF-α*–308 G/A (rs1800629) and –238 G/A (rs361525); *IL-4* –1098 T/G (rs 2243248) and –590 T/C (rs2243250); *IL-6* –174 G/C (rs1800795) and nt565 G/A (rs1800797); and *IL-10* –1082 G/A (rs1800896), –819 C/T (rs1800871) and –592 C/A (rs1800872) gene polymorphism (Table 1).

Table 1. Single nucleotide polymorphisms showing allele and genotype frequencies in patients with systemic sclerosis and healthy controls.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	*p*-value
IL1β -511 (rs16944)	Alleles	T	33 (71.7)	80 (50.0)	**0.011***
		C	13 (28.3)	80 (50.0)	**0.011***
	Genotypes	TT	14 (60.9)	29 (36.3)	0.054
		TC	5 (21.7)	22 (27.4)	0.789
		CC	4 (17.4)	29 (36.3)	0.128
IL1β +3962 (rs1143634)	Alleles	C	28 (60.9)	129 (80.6)	**0.01***
		T	18 (39.1)	31 (19.4)	**0.01***
	Genotypes	CC	11 (47.8)	56 (70.0)	0.081
		CT	6 (26.1)	17 (21.2)	0.585
		TT	6 (26.1)	7 (8.8)	0.068
IL4Rα +1902 (rs1801275)	Alleles	G	9 (19.6)	23 (14.4)	0.488
		A	37 (80.4)	137 (85.6)	0.488
	Genotypes	GG	2 (8.7)	0 (0.0)	**0.048***
		GA	5 (21.7)	23 (28.8)	0.602
		AA	16 (69.6)	57 (71.2)	1.000

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IL12 -1188 (rs3212227) [deviation from HWE, therefore excluded]	Alleles	C	16 (34.8)	94 (58.8)	**0.005***
		A	30 (65.2)	66 (41.2)	**0.005***
	Genotypes	CC	0 (0.0)	35 (43.8)	**0.000***
		CA	16 (69.6)	24 (30.0)	**0.001***
		AA	7 (30.4)	21 (26.2)	0.791
TGFβ1 codon10 (rs1982073)	Alleles	C	23 (50.0)	81 (50.6)	1.000
		T	23 (50.0)	79 (49.4)	1.000
	Genotypes	CC	0 (0.0)	1 (1.2)	1.000
		CT	23 (100.0)	79 (98.8)	1.000
		TT	0 (0.0)	0 (0.0)	-
TGFβ1 codon25 (rs1800471)	Alleles	G	35 (76.1)	96 (60.0)	0.056
		C	11 (23.9)	64 (40.0)	0.056
	Genotypes	GG	12 (52.2)	16 (20.0)	**0.006***
		GC	11 (47.8)	64 (80.0)	**0.006***
		CC	0 (0.0)	0 (0.0)	-
TGFβ1 codon10, codon25	Haplotypes	CG	12 (26.1)	17 (10.6)	**0.014***
		TG	23 (50.0)	79 (49.4)	1.000
		CC	11 (23.9)	64 (40.0)	0.056
TNFα -308 (rs1800629)	Alleles	G	42 (91.3)	103 (64.4)	**0.000***
		A	4 (8.7)	57 (35.6)	**0.000***
	Genotypes	GG	19 (82.6)	23 (28.8)	**0.000***
		GA	4 (17.4)	57 (71.2)	**0.000***
		AA	0 (0.0)	0 (0.0)	-
TNFα -238 (rs361525)	Alleles	G	27 (58.7)	137 (85.6)	**0.000***
		A	19 (41.3)	23 (14.4)	**0.000***
	Genotypes	GG	4 (17.4)	57 (71.2)	**0.000***
		GA	19 (82.6)	23 (28.8)	**0.000***
		AA	0 (0.0)	0 (0.0)	-
TNFα -308, -238	Haplotypes	GG	23 (50.0)	80 (50.0)	1.000
		AG	4 (8.7)	57 (35.6)	**0.000***
		GA	19 (41.3)	23 (14.4)	**0.000***
IL4 -1098 (rs 2243248)	Alleles	T	35 (76.1)	138 (86.2)	0.112
		G	11 (23.9)	22 (13.8)	0.112
	Genotypes	TT	14 (60.9)	58 (72.5)	0.309
		TG	7 (30.4)	22 (27.5)	0.796
		GG	2 (8.7)	0 (0.0)	**0.048***
IL4 -590 (rs2243250)	Alleles	C	38 (82.6)	109 (68.1)	0.065
		T	8 (17.4)	51 (31.9)	0.065
	Genotypes	CC	15 (65.2)	43 (53.7)	0.353
		CT	8 (34.8)	23 (28.8)	0.611
		TT	0 (0.0)	14 (17.5)	**0.036***
IL4 -33 (rs2070874)	Alleles	T	9 (19.6)	52 (32.5)	0.102
		C	37 (80.4)	108 (67.5)	0.102
	Genotypes	TT	3 (13.05)	15 (18.8)	0.757
		TC	3 (13.05)	22 (27.4)	0.180
		CC	17 (73.9)	43 (53.8)	0.098
IL6 -174 (rs1800795)	Alleles	G	38 (82.6)	144 (90.0)	0.193
		C	8 (17.4)	16 (10.0)	0.193
	Genotypes	GG	18 (78.3)	64 (80.0)	1.000
		GC	2 (8.7)	16 (20.0)	0.350
		CC	3 (13.0)	0 (0.0)	**0.01***
IL6 nt565 (rs1800797)	Alleles	G	40 (87.0)	145 (90.6)	0.580
		A	6 (13.0)	15 (9.4)	0.580
	Genotypes	GG	20 (87.0)	65 (81.2)	0.757
		GA	0 (0.0)	15 (18.8)	**0.021***
		AA	3 (13.0)	0 (0.0)	**0.01***

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IL10 -1082 (rs1800896)	Alleles	A	32 (69.6)	88 (55.0)	0.091
		G	14 (30.4)	72 (45.0)	0.091
	Genotypes	AA	9 (39.1)	8 (10.0)	**0.002***
		AG	14 (60.9)	72 (90.0)	**0.002***
		GG	0 (0.0)	0 (0.0)	-
IL10 -819 (rs1800871)	Alleles	C	23 (50.0)	103 (64.4)	0.088
		T	23 (50.0)	57 (35.6)	0.088
	Genotypes	CC	6 (26.1)	23 (28.8)	1.000
		CT	11 (47.8)	57 (71.2)	**0.047***
		TT	6 (26.1)	0 (0.0)	**0.000***
IL10 -592 (rs1800872)	Alleles	A	19 (41.3)	57 (35.6)	0.493
		C	27 (58.7)	103 (64.4)	0.493
	Genotypes	AA	2 (8.7)	0 (0.0)	**0.048***
		AC	15 (65.2)	57 (71.2)	0.611
		CC	6 (26.1)	23 (28.8)	1.000
IL10 -1082, -819, -592	Haplotypes	ATA	17 (37.0)	50 (31.2)	0.479
		ACC	15 (32.6)	38 (23.8)	0.252
		GCC	14 (30.4)	72 (45.0)	0.091

Allele, genotype and haplotype frequencies are presented as absolute numbers with percentage in parentheses; rs - refSNP cluster ID number;
*Mean difference is significant at the indicated *p*-value

As polymorphism in *IL-12* –1188 C/A did not obey HWE, it was excluded from the study. While HWE deviations can signal genotyping errors or population stratification, in our case, they are likely due to the small sample size and the specific characteristics of the SSc population.

IL-1β Cytokine Gene Polymorphism

Significant differences in allele distributions were found between the patients with SSc and controls for *IL-1β* +3962. The T allele was significantly more common in patients than in controls (OR 2.675, 95% CI 1.315-5.442; $p<0.05$). The T allele of *IL-1β* -511 also showed a trend towards association with SSc (OR 2.538, 95% CI 1.245-5.177; $p<0.05$). No significant difference in their genotype distribution was found between patients with SSc and controls (all $p>0.05$) (Table 2).

TNF-α Cytokine Gene Polymorphism

The *TNF-α* -308 G allele frequency was significantly increased in patients with SSc compared with the healthy volunteers (91.3% vs 64.4%; $p<0.001$; OR 5.811, 95% CI 1.982- 17.032). The GG genotype at *TNF-α* -308 was also significantly increased in the SSc patients (82.6% vs 28.8%; $p<0.001$; OR 11.772, 95% CI 3.61-38.384). Carriage of the *TNF-α* -238 A allele was significantly more common among patients with SSc than among control subjects (41.3% vs 14.4%; $p<0.001$; OR 4.192, 95% CI 2.011–8.737). The GA genotype frequency at *TNF-α* -238 was also significantly higher in patients than in controls (82.6% vs 28.8%; $p<0.001$; OR 11.772, 95% CI 3.61-38.384). Haplotype analysis showed that specific (-308G -238A) haplotype was observed more often in the SSc patients than in controls ($p<0.001$) (Table 3).

Table 2. IL-1β Cytokine Gene Polymorphism association with systemic sclerosis.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	*p*-value	Odds Ratio	95% CI
IL-1β -511 (rs16944)	Alleles	T	33 (71.7)	80 (50.0)	**0.011***	2.538	1.245 - 5.177
		C	13 (28.3)	80 (50.0)	**0.011***	0.394	0.193 - 0.803
	Genotypes	TT	14 (60.9)	29 (36.3)	0.054	2.736	1.054 - 7.098
		TC	5 (21.7)	22 (27.4)	0.789	0.732	0.242 - 2.213
		CC	4 (17.4)	29 (36.3)	0.128	0.370	0.115 - 1.194
IL-1β +3962 (rs1143634)	Alleles	C	28 (60.9)	129 (80.6)	**0.01***	0.374	0.184 - 0.760
		T	18 (39.1)	31 (19.4)	**0.01***	2.675	1.315 - 5.442
	Genotypes	CC	11 (47.8)	56 (70.0)	0.081	0.393	0.152 - 1.013
		CT	6 (26.1)	17 (21.2)	0.585	1.308	0.447 - 3.828
		TT	6 (26.1)	7 (8.8)	0.068	3.681	1.096 - 12.36

Allele and genotype frequencies are presented as absolute numbers with percentage in parentheses;
rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *Mean difference is significant at the indicated *p*-value

Table 3. TNF-α Cytokine Gene Polymorphism association with systemic sclerosis.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	*p*-value	Odds Ratio	95% CI
TNF-α -308 (rs1800629)	Alleles	G	42 (91.3)	103 (64.4)	**0.000***	5.811	1.982 - 17.032
		A	4 (8.7)	57 (35.6)	**0.000***	0.172	0.059 - 0.504
	Genotypes	GG	19 (82.6)	23 (28.8)	**0.000***	11.772	3.61 - 38.384
		GA	4 (17.4)	57 (71.2)	**0.000***	0.085	0.026 - 0.277
		AA	0 (0.0)	0 (0.0)	-	-	-
TNF-α -238 (rs361525)	Alleles	G	27 (58.7)	137 (85.6)	**0.000***	0.239	0.114 - 0.497
		A	19 (41.3)	23 (14.4)	**0.000***	4.192	2.011 - 8.737
	Genotypes	GG	4 (17.4)	57 (71.2)	**0.000***	0.085	0.026 - 0.277
		GA	19 (82.6)	23 (28.8)	**0.000***	11.772	3.61 - 38.384
		AA	0 (0.0)	0 (0.0)	-	-	-
TNF-α -308, -238	Haplotypes	GG	23 (50.0)	80 (50.0)	1.000	1.000	0.519 - 1.927
		AG	4 (8.7)	57 (35.6)	**0.000***	0.172	0.059 - 0.504
		GA	19 (41.3)	23 (14.4)	**0.000***	4.192	2.011 - 8.737

Allele, genotype and haplotype frequencies are presented as absolute numbers with percentage in parentheses;
rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *Mean difference is significant at the indicated *p*-value

Table 4. IL-10 Cytokine Gene Polymorphism association with systemic sclerosis.

Cytokine polymorphism			SSc (n=23)	HC (n=80)	p-value	Odds Ratio	95% CI
IL-10 -1082 (rs1800896)	Alleles	A	32 (69.6)	88 (55.0)	0.091	1.870	0.928 - 3.77
		G	14 (30.4)	72 (45.0)	0.091	0.535	0.265 - 1.078
	Genotypes	AA	9 (39.1)	8 (10.0)	**0.002***	5.786	1.904 17.577
		AG	14 (60.9)	72 (90.0)	**0.002***	0.173	0.057 - 0.525
		GG	0 (0.0)	0 (0.0)	-	-	-
IL-10 -819 (rs1800871)	Alleles	C	23 (50.0)	103 (64.4)	0.088	0.553	0.285 - 1.073
		T	23 (50.0)	57 (35.6)	0.088	1.807	0.932 - 3.504
	Genotypes	CC	6 (26.1)	23 (28.8)	1.000	0.875	0.306 - 2.497
		CT	11 (47.8)	57 (71.2)	**0.047***	0.370	0.143 - 0.957
		TT	6 (26.1)	0 (0.0)	**0.000***	0.739	0.580 - 0.942
IL-10 -592 (rs1800872)	Alleles	A	19 (41.3)	57 (35.6)	0.493	1.272	0.651 - 2.485
		C	27 (58.7)	103 (64.4)	0.493	0.786	0.402 - 1.537
	Genotypes	AA	2 (8.7)	0 (0.0)	**0.048***	0.913	0.805 - 1.036
		AC	15 (65.2)	57 (71.2)	0.611	0.757	0.282 - 2.026
		CC	6 (26.1)	23 (28.8)	1.000	0.875	0.306 - 2.497
IL-10 -1082, -819, -592	Haplotypes	ATA	17 (37.0)	50 (31.2)	0.479	1.290	0.650 - 2.560
		ACC	15 (32.6)	38 (23.8)	0.252	1.553	0.759 - 3.179
		GCC	14 (30.4)	72 (45.0)	0.091	0.535	0.265 - 1.078

Allele, genotype and haplotype frequencies are presented as absolute numbers with percentage in parentheses;
rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *Mean difference is significant at the indicated *p*-value

IL-10 Cytokine Gene Polymorphism

There were significant differences in the genotype frequencies of the *IL-10* -1082 A/G, - 819 C/T and -592 C/A polymorphisms between SSc patients and healthy volunteers. We found a higher frequency of the AA genotype at *IL-10* -1082 (39.1% vs 10%; $p<0.01$), TT genotype at *IL-10* -819 (26.1% vs 0%; $p<0.001$) and the AA genotype at *IL-10* -592 (8.7% vs 0%; $p<0.05$) in SSc patients than in the healthy volunteers. We also found that the ACC and ATA haplotypes were more frequent in SSc patients compared to healthy volunteers, but the difference was not statistically significant (Table 4).

In addition to these results, significantly higher frequencies of genotypes were also observed in SSc patients as compared to controls in *TGF-β1* codon25 GG genotype (52.2% vs 20.0%; $p<0.01$) and *IL-6* nt565 AA genotype (13% vs 0%; $p<0.05$). *IL-6* gene polymorphism showed near complete linkage disequilibrium between the –174 G and nt565 G alleles.

DISCUSSION

Cytokine production and release are key events in SSc pathogenesis as they are involved in T and B cell activa-

tion leading to inflammation, auto-antibodies production, microvascular damage and fibrosis [21]. The Th1/Th2/Th17/Treg balance is one of the hallmarks of SSc pathogenesis, as the Th2 and Th17 cytokines response leads to tissue fibrosis, whereas Th1 and Th17 cytokines promote inflammation in SSc patients.

IL-1α and *IL-1β* are proinflammatory cytokines involved in a number of autoimmune diseases. Patients who have SSc have increased circulating levels of *IL-1α* and *IL-1β*. Genetic associations with *IL-1β* have been investigated in patients with SSc and significant associations of the *IL-1β* -31 C and *IL-1β* -511 T alleles have been found [17]. Our results provide evidence suggesting that the T alleles of *IL-1β* -511 and *IL-1β* +3962 are associated with SSc in our population. Polymorphism in the human *IL-1β* gene has been reported to influence cytokine expression [22]. *IL-1β* stimulates the production of prostaglandin E2, which is an important cofactor for the induction of T-helper lymphocyte activity towards Th2 direction. A shift towards the Th2 system has been indicated in SSc [11].

Changes in *IL-1β* expression levels may reflect the genetic variation in *IL-1β* gene. The findings on biological roles of *IL-1β* polymorphisms, however, have not been consistent across studies. TT genotype of *IL-1β* -511 has been associated with higher gastric mucosa *IL-1β* levels in *Helicobacter pylori* positive population [23]. On the other hand, subjects with CC genotype showed an increased release of *IL-1β* from mononuclear cells after stimulation with lipopolysaccharide [24]. Recent studies suggest that the functional role of *IL-1β* -511 may depend on *IL-1β* promoter region haplotypes including *IL-1β* -511 [25]. Although the findings are inconsistent, these previous studies suggest that *IL-1β* -511 could affect the expression levels of *IL-1β*. On the other hand, the influence of *IL-1β* +3962 on *IL-1β* expression levels has not been previously reported. Polymorphisms in *IL-1β*, particularly SNPs *IL-1β* +3962 and *IL-1β* -511, have been identified as risk factors for susceptibility, progression, and severity of periodontal disease across various populations [26,27]. Elevated *IL-1β* levels in gingival crevicular fluid, saliva, and serum of periodontitis patients further support these associations [27]. Additionally, existing literature links inflammation associated with SSc etiology to the development of oral conditions like periodontitis [28]. Together, these findings suggest that *IL-1β* gene polymorphisms may contribute to the development of SSc in our study population.

A significant association of *TNF-α* -308 G allele and *TNF-α* -238 A allele with SSc was observed in this study. *TNF-α*, a member of *TNF*-superfamily, is a potent pro-inflammatory cytokine which affects different aspects of immune response, cell growth, differentiation and activation [29]. Due to its broad spectrum of pro- inflammatory functions *TNF-α* has been implicated in the pathogenesis of many immune disorders including all connective tissue diseases [29]. Increased production of *TNF-α* by PBMCs as well as elevated serum concentrations of *TNF-α* have been demonstrated SSc patients [30]. The enhanced production of *TNF-α* by PBMCs of SSc patients is associated with increased synthesis of *TNF-α* mRNA indicating increased expression of the *TNF-α* gene in SSc patients [31]. Moreover, elevated concentrations of *TNF-α* have been demonstrated in bronchoalveolar lavage fluid of SSc patients with interstitial lung disease [32]. Similarly, elevated serum *TNF-α* concentration in SSc patients were found in SSc patients with pulmonary fibrosis [33]. Recent studies have revealed inconsistent results regarding correlation of *TNF-α* polymorphisms with periodontitis susceptibility [34].

In our study, despite the patients and controls being of the same ethnic origin and from the same geographic region, we identified a strong association between the *TNF-α* -308 G allele and SSc. The functional significance and transcriptional impact of this allele remain a topic of debate, as some studies have found no direct link between *TNF-α* -308 polymorphisms and *TNF-α* production [35]. However, it is possible that an unidentified gene in linkage disequilibrium with the *TNF-α* -308 G allele may play a role in the increased susceptibility to SSc observed in individuals carrying this allele. In contrast, the *TNF-α* -238 A allele and GA genotype have also been previously associated with SSc [36]. Collectively, these findings suggest that *TNF-α* gene polymorphisms contribute to the pathogenesis of SSc in our study population.

The *IL-10* gene promoter region contains several SNPs including –1082 G/A, –819 C/T and –592 C/A in the transcription factor-binding region. Alleles of all three polymorphisms are in linkage disequilibrium, giving rise to only three major allele combinations out of possible eight in Caucasian populations: the GCC haplotype is responsible for higher *IL-10* secretion, whereas ACC and ATA haplotypes are associated with its lower production. Although we observed significant differences in the genotypes of the three *IL-10* SNPs, no significant difference in haplotype (GCC/ATA/ACC) distribution between SSc patients and healthy individuals was observed. The G allele at −1082, and haplotypes containing this allele, have been associated with high *IL-10* production, while the A allele and the ATA haplotype have been associated with low *IL-10* production [37]. Our study does not show a possible correlation between *IL-10* SNPs and its production in SSc patients. However, a significant association of *IL-10* SNPs have recently been shown with chronic periodontitis [38].

The importance of *TGF-β* in SSc pathogenesis has been demonstrated well. *TGF-β*, its receptor, and down-

stream signaling molecules are expressed at increased levels in affected organs in SSc. *TGF-β* activates dermal fibroblasts leading to increased production of extracellular matrix. Given the importance of *TGF-β* in SSc, it has been hypothesized that polymorphisms in its gene may contribute to SSc susceptibility. However, there is a paucity of studies in this direction and the findings have been conflicting [39-41]. In this study we found no association between the SNP in *TGF-β1* codon10 and SSc. However, significant differences were observed in *TGF-β1* codon25 genotypes between patients and controls. Given the strong linkage disequilibrium among the SNPs in this gene, it is difficult, if not impossible, to assess which, if any, of these SNPs is truly responsible for the quantitative variation in *TGF-β1* level. Possibly, particular alleles at these loci additively (or interactively) influence the quantitative (and possibly qualitative) expression of this cytokine.

The significant association between *IL-1β* (-511, +3962) and *TNF-α* (-308, -238) SNPs with SSc suggests a genetic predisposition to the disease, highlighting their potential as biomarkers for early diagnosis, risk assessment, and targeted therapies. These SNPs may contribute to SSc pathogenesis by dysregulating key inflammatory cytokines, promoting fibrosis and vascular damage. Additionally, they could help explain disease heterogeneity, offering insights into the severity of SSc and enabling better patient stratification. Given their role in other autoimmune conditions, these findings also provide broader implications for shared pathogenic mechanisms and therapeutic strategies, with the potential for inclusion in genetic screening panels for at-risk individuals.

Our study is limited by a small sample size and a restricted number of SNPs, as genetic susceptibility to SSc likely involves a broader combination of genes, along with environmental and epigenetic factors. Additionally, we did not adjust *p*-values for multiple testing, which complicates the determination of statistical significance and requires further investigation. While our findings suggest a potential association between *IL-1β* and *TNF-α* SNPs and SSc, larger studies are needed to confirm these results. The higher proportion of females in the SSc patient group compared to the control group reflects the well-known female predominance in systemic sclerosis [42], which is more common in women, especially in younger and middle-aged adults. While this sex distribution aligns with existing epidemiological data, the small sample size and sex imbalance may reduce the study's statistical power and affect the generalizability of the results. Larger, more balanced studies are needed to confirm these findings and explore potential sex-specific genetic associations.

Future research should explore the functional roles of these SNPs through *in vitro* expression studies to evaluate their effects on cytokine production and immune cell activation. Employing genome-editing technologies like CRISPR-Cas9 could also provide insights into how these SNPs influence gene expression and cellular responses in relevant immune cells. Longitudinal cohort studies in diverse populations would further elucidate how these variants correlate with disease progression and treatment outcomes. These approaches would offer valuable insights into the mechanisms underlying SSc and help identify potential therapeutic targets, providing clearer directions for future studies in this field.

CONCLUSION

Our study found a significant association between *IL-1β* -511, +3962, and *TNF-α* -308, -238 SNPs and SSc in our population, consistent with previous research linking these SNPs to SSc susceptibility and progression. Larger, more balanced studies are needed to confirm these associations. Future research should focus on the functional roles of these SNPs through *in vitro* studies, genome editing, and longitudinal cohort studies to better understand their impact on disease progression and treatment outcomes.

Author Statement

The manuscript represents original, unpublished material not under editorial consideration elsewhere, and that ethical guidelines were followed in the conduct of the research.

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None

Conflict of Interest

The authors have no conflict of interest to disclose.

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