

## The IL - 6 promoter polymorphism is associated with disease activity and disability in systemic sclerosis

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### Abstract

Systemic sclerosis (SSc) is a rare, autoimmune disease characterized by cutaneous and visceral fibrosis. Interleukin-6 (IL-6) is involved in the pathogenesis of many immune-mediated diseases. IL-6 plays an important role in the initiation and promotion of fibrosis. The polymorphism in the position -174 (G/C) of the promoter region of the IL-6 gene (IL-6 pr) may alter the expression of the gene. Complete linkage disequilibrium was observed between the -174 and -597 alleles. The aim of this study is to investigate the possible influence of -597 (-174) IL-6 pr polymorphism on the susceptibility and/or the clinical course of SSc in Romanian population. Genotyping of -597 variant was performed by an RFLP method on 20 SSc patients and 26 healthy subjects. Patients having the homozygous GG (-597) genotype had higher disease activity and disability scores than heterozygous GA patients: the European Scleroderma Study Group (EScSG) disease activity score was  $5.0 \pm 3.3$  in homozygous GG subjects vs.  $2.4 \pm 3.6$  in heterozygous GA patients ( $p < 0.05$ ), and the Disability Index of the Health Assessment Questionnaire (HAQ-DI) was  $1.42 \pm 1.04$  in homozygous GG subjects vs.  $0.53 \pm 0.55$  in heterozygous GA patients ( $p < 0.05$ ). No difference was observed in the distribution of allele frequencies between SSc patients and healthy controls. Conclusions: The GG homozygosis was found to be associated with a higher degree of illness activity and disability in SSc patients. No statistically significant differences were found between SSc patients and healthy controls with respect to the -597 allele distribution.

**Keywords:** interleukin-6 (IL-6) • gene polymorphism • systemic sclerosis (SSc) • fibrosis • cytokine

### Introduction

Systemic sclerosis (SSc) is a rare autoimmune disease (its estimated prevalence is less than 300 cases per million in the USA population) [1]. To present,

there are no epidemiological studies regarding SSc in Romanian population. The most characteristic feature of SSc is cutaneous and visceral fibrosis. Currently, two different disease subsets are recognized in SSc: limited cutaneous SSc (l-SSc) and diffuse cutaneous SSc (d-SSc), according to the extension of the cutaneous involvement. Recent data provide strong evidence for cytokine (CK) involvement

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in the initiation and promotion of fibrosis. Among these cytokines, the most studied are: transforming growth factor- $\beta$  (TGF- $\beta$ ), connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), and interleukins 1 $\alpha$  (IL-1  $\alpha$ ), 4 (IL-4), 6 (IL-6), 10 (IL-10), 13 (IL-13), and 17 (IL-17) [2-6]. TGF- $\beta$ , CTGF, PDGF and IL-6 are powerful inducers of collagen production and fibroblast proliferation. The dermal fibroblasts from SSc patients secrete these CK in higher amounts than normal fibroblasts, mostly because of stimulation by IL-1 $\alpha$  [4, 5]. IL-6 seems to be also implicated in angiogenesis and neovascularization. There are studies suggest that decreased angiogenesis may be responsible for aberrant vascular proliferation and ischemic manifestations seen in SSc. [for review see ref.7].

There is evidence that serum levels of IL-6 are elevated in patients with SSc [8] and these were correlated with the extension of cutaneous affection, assessed by the modified Rodnan Score [9]. The peripheral blood mononuclear cells of SSc patients produce increased amounts of IL-6 [10, 11]. The cultured dermal fibroblasts from SSc patients synthesize elevated levels of IL-6 [12, 13]. The increased IL-6 production seems to be the result of stimulation *via* IL-1 $\alpha$  and PDGF [12].

Characterization of the IL-6 promoter region (IL-6pr) has revealed the presence of several genetic polymorphisms: -572 [14], -174 [15, 16, and 17], -597 [18, 19], -596 [20], -622 [21]. A G/C polymorphism at the -174 position, located immediately next to the multiresponsive element, has been described [22]. This single nucleotide polymorphism (SNP) regulates the transcription of the IL-6 gene and correlates with plasma IL-6 levels in healthy Caucasians; both GG homozygotes and GC heterozygotes have been shown to have higher plasma IL-6 levels, higher IL-6 gene transcriptional activity and higher inducible IL-6 responses than CC homozygotes [22]. Complete linkage disequilibrium has been observed between the -597 and -174 alleles [18, 19].

In this study we tried to investigate the possible association between the -597 SNP of the IL-6 promoter and either the susceptibility to SSc or the clinical course of this disease. We found that the GG (-597) homozygosis was associated with a higher degree of illness activity and greater disability in SSc patients as compared to the GC (-597) heterozygotes. The -597 SNP of the IL-6 promoter was not correlated with the susceptibility to SSc on the studied group.

## Materials and methods

### Subjects

The study was performed on two groups of subjects: a group of 20 patients (18 women and 2 men) diagnosed with SSc, and a group of 26 healthy blood donors of the same ethnic origin and geographic area. For SSc subset analysis, we used the criteria of LeRoy *et al.* [23] to divide patients into diffuse cutaneous SSc (d-SSc) and limited cutaneous SSc (l-SSc) subtypes. All the peripheral blood samples were obtained after informed consent and the study was approved by the local Ethics Committee. The mean disease duration was  $6.8 \pm 6.2$  years and the average age of subjects was  $44.4 \pm 10.7$  years in patients and  $40.2 \pm 11.6$  in controls. Patients were evaluated in detail with a standard clinical and visceral involvement protocol, which permitted us to compute the disease activity score [24] and the disease severity score [25] as recommended by the European Scleroderma Study Group (EScSG). Disease activity was also globally assessed by the clinical investigator, first as either high or low and, second, on a 100 mm visual analog scale (VAS), where 0 stands for inactive disease and 100 stands for highest possible activity. All the patients completed the Disability Index of the Health Assessment Questionnaire (HAQ-DI) [26].

### PCR-RFLP analysis

DNA was extracted using Wizard Genomic Purification kit (PROMEGA, Madison, Wisconsin, USA) by standard procedures as recommended by the manufacturer. Restriction fragment length polymorphism (RFLP) was performed with the -597 IL-6 promoter instead of -174 SNP itself, since both sites are at complete linkage disequilibrium [19]. The polymorphism locus was amplified using the forward primer 5'GGAGTCACACACTCCACT 3' and the reverse 5'CTGATTGGAAACCTTATTAAG 3'. PCR cycling conditions were as follows: an initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 30 s, 54°C for 40 s and 72°C for 50 s; one cycle at 72°C for 10 min. Amplification yielded a band of 525 bp. An aliquot of products was digested at 37°C for 1 h with Fok I (Roche Applied Science, Basel, Switzerland). The digested fragments of 525 bp, 459 and 66 bp were separated by 8% polyacrylamide (PAA) gel electrophoresis (Sigma-Aldrich Corporation, St Louis-Missouri, USA). Gels were stained with ethidium bromide and products were visualized with ultraviolet light (UV).

## Statistical analysis

Differences of genotype frequencies between healthy controls and SSc patients were analyzed by the  $\chi^2$  test. The results obtained for each genotype were compared with those predicted in a population by Hardy-Weinberg equilibrium. Association between genotypes and sex, disease subsets, or the presence of various organ-system involvements, was also analyzed by the  $\chi^2$  test. Disease activity and severity scores and the HAQ-DI [26] among patients with different genotypes were compared by the Mann Whitney U test. The analysis was carried out using the SPSS statistical software, release 11.0 (SPSS, Chicago, IL); p values less than 0.05 were considered statistically significant.

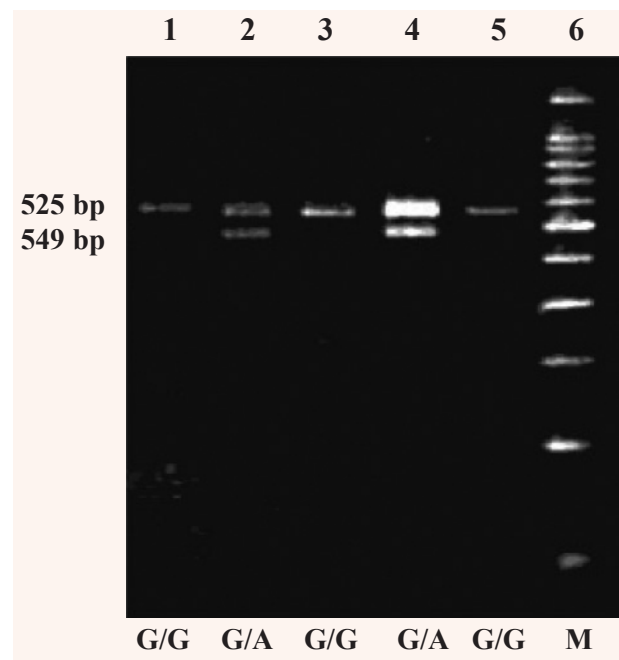
## Results

The PCR-RFLP analysis of the polymorphism is presented in Fig 1. We also used the DNA sequencing method for 9 samples and the same results as by the PCR-RFLP method were obtained. Frequencies of the alleles -597 G/G, -597 G/A and -597 A/A, were 54%, 45% and 0% in healthy controls, and 45%, 54% and 0% in the SSc patients group, respectively. No statistically significant differences were found between SSc patients and healthy controls referring to the -597 allele distribution.

In order to establish whether there was an association -597 IL-6pr polymorphism with the clinical course of the disease, the -597 IL-6pr variants in the different clinical subsets d-SSc and l-SSc were examined. No statistical differences in the distribution of IL-6pr genotypes were found between the two disease subsets. However, the GG (-597) homozygote patients had higher disease activity scores and greater disability than the GA (-597) heterozygote patients (Table 1).

## Discussion

The mechanisms by which IL-6 contributes to the development of SSc disease are not fully understood, but elevated serum levels of IL-6 were found to be associated with total skin thickness score [9], with the occurrence of pulmonary fibrosis and



**Fig. 1** The PCR-RFLP analysis of the -597 polymorphism for the IL-6 gene in 8% PAA gel electrophoresis. Samples from lanes 1–5 are from different subjects. The size of the digested PCR products from different IL-6pr (-597) genotypes are shown. Lane 6 represents the control for molecular size markers (M).

decrease in pulmonary vital capacity [8]. The consequences of lung exposure to IL-6 excess have also been studied in a rat model in which the induced local over expression of IL-6 led to lymphocytic alveolitis and destructive interstitial pneumonia [27]. These data suggest that even if the polymorphism of IL-6pr is not responsible for the development of the SSc, it may still play a role in the mechanisms of this disease.

It has been shown that the transcriptional activity of the IL-6 gene and the plasma levels of IL-6 are associated with a single G/C base exchange polymorphism sited at the -174 position [22]. This leads to variations in expression levels and may influence susceptibility to different autoimmune diseases. The IL-6 -174G/C polymorphism has already been found to be associated with some autoimmune and inflammatory diseases, such as diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, and juvenile chronic arthritis [16, 17, 21, 22]. Interleukin promoter polymorphisms have been associated not only with susceptibility but also with outcome in autoimmune diseases [27]. The GG

**Table 1** The GG (-597) homozygosis was associated with a higher degree of illness activity and greater disability in SSc patients, as compared to GA (-597) heterozygote patients.

	Genotype		Statistical significance
	GG (n = 9)	GA (n = 11)	
Number (n) of patients with disease subsets: d-SSc-l-SSc	4/5	5/6	NS*
Number (n) of patients with higher/lower degree of disease activity	7/2	2/9	p < 0,05*
Global disease activity score (VAS)	59.3 ± 13.5	37.8 ± 24.7	p < 0,05†
Activity score (EScSG)	5.0 ± 3.3	2.4 ± 3.6	p < 0,05†
Disability score (HAQ)	1.42 ± 1.04	0.53 ± 0.55	p < 0,05†
Severity score (Medsger)	7.83 ± 1.94	8.43 ± 3.21	NS†

Statistical significance was assessed by the  $\chi^2$  test (†) and the U Mann-Whitney Test (\*); NS = not significant (p > 0.05); EScSG (European Scleroderma Study Group) [24]; HAQ-DI (Health Assessment Questionnaire Disability Index) [26]; Severity Score Medsger [25]; VAS = visual analog scale (0 - no activity; 100 - as active as possible); d-SSc- diffuse systemic sclerosis; l-SSc- limited systemic sclerosis

homozygote and the GC heterozygote have higher plasma IL-6 levels than the CC homozygote.

Since the G allele seems to correlate positively with the plasma level of IL-6, we suggest that the presence of this allele may be a risk factor for the development of fibrosis associated with a high IL-6 concentration in SSc. Due to the total linkage disequilibrium, the -174 G allele is always associating the G allele in the position -597, allowing us to expect that subjects homozygous for the G allele in the -597 position of the IL-6pr would have a higher risk to develop SSc than the -597 AA homozygote patients [19, 8].

To our knowledge this is the first study on the possible association between -597/-174 IL-6pr polymorphism and SSc. We analyzed differences between patients having the homozygous GG (-597) genotype and patients having the heterozygous GA (-597) genotype, and we found that the homozygous GG (-597) patients had higher disease activity and higher disability scores than the heterozygous GA patients, using the European Scleroderma Study Group (EScSG) disease activity score and the Disability Index of the Health Assessment Questionnaire (HAQ-DI). These data suggest that the G allele is associated with a greater

disease activity and a worse disease outcome, and if this association could be confirmed on a larger patient group, it might be used as a factor for prognostic. There was no other significant association between the studied polymorphism and the demographic or epidemiological data of the patients (sex, age of onset, organ-system involvement and disease subset). When comparing the SSc patient group with the healthy control group, no significant difference in the allele frequency was observed.

## Conclusions

The GG (-597) homozygosis was found to be associated with a higher degree of illness activity and greater disability in SSc patients, as compared to GA (-597) heterozygote patients. No statistically significant differences were found between SSc patients and healthy controls with respect to the -597 allele distribution. However, further work on a larger number of subjects is needed to statistically support a definitive conclusion about the correlation between allele distribution and SSc susceptibility and clinical course.

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