

Analysis of the 5' flanking region of the interleukin 10 gene in patients with systemic sclerosis

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Objectives. Fibrosis, a feature of systemic sclerosis (SSc), is more severe in the diffuse compared with the limited disease variant. Interleukin 10 (IL-10) is an anti-inflammatory cytokine which reduces type 1 collagen mRNA levels in human fibroblasts. The 5' flanking region of the IL-10 gene is highly polymorphic, with three single base pair substitutions at position -1082(G/A), -819(C/T) and -592(C/A), which results in differential IL-10 production. The GCC/GCC genotype is associated with high IL-10 production while the ATA/ATA genotype with low production. We postulated that there would be a difference in IL-10 polymorphisms in patients with limited (lSSc) and diffuse (dSSc) disease.

Methods. Patients with limited (lSSc, $n = 89$) or diffuse (dSSc, $n = 51$) disease plus controls ($n = 94$) were recruited. DNA was isolated from peripheral blood and polymorphisms analysed using amplification refractory mutation system (ARMS) polymerase chain reaction (PCR).

Results. dSSc patients were less likely to carry the genotype indicative of high IL-10 production when compared with controls (controls vs dSSc; 29 vs 4%, $\chi^2 = 15.7$, 5 df, $P = 0.005$) and lSSc patients (lSSc vs dSSc; 21 vs 4%, $\chi^2 = 17.5$, 5 df, $P = 0.002$). There was no difference between control and lSSc patients. While there was no difference between controls and lSSc haplotypes, the GCC haplotype distribution did differ significantly between controls and dSSc patients (controls vs dSSc; 54 vs 36%, $\chi^2 = 11.2$, 2 df, $P = 0.001$). A significant difference was also observed between lSSc and dSSc haplotype distribution (lSSc vs dSSc; 48 vs 36%, $\chi^2 = 13.5$, 2 df, $P < 0.001$).

Conclusion. We demonstrate that IL-10 genotypes associated with high IL-10 production are under-represented in dSSc. This may have implications in the disease pathology.

KEY WORDS: Systemic sclerosis, Polymorphisms, Interleukin 10.

Systemic sclerosis or scleroderma (SSc) describes several distinct disorders of which the diffuse (dSSc) and limited (lSSc) variants are the best characterized. The clinical features and outcome of the two differ, although rarely there may be an overlap. In both there are microvascular changes, excess deposition of extracellular matrix (ECM) and immunological abnormalities of which characteristic autoantibody production is best described. A fundamental difference between lSSc and dSSc is the extent of ECM deposition. Excess ECM is more a

feature of dSSc, whereas in lSSc microvascular changes predominate [1].

ECM production by fibroblasts in SSc is modulated and regulated by cytokines [2]. The biological properties of TGF β 1 and the finding of excess TGF β 1 at affected sites in dSSc suggest its key role in driving the fibroblasts in SSc [3]. Interleukin 10 (IL-10) is an anti-inflammatory cytokine secreted by monocytes and lymphocytes [4]. As well as having anti-inflammatory properties, *in vitro* it inhibits collagen production and secretion from both

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normal and SSc fibroblasts [5]. IL-10 has not been sought in affected SSc tissue, but serum levels have been measured [6–8]. Serum IL-10 levels appear not to be significantly higher in SSc.

There is an up to 10-fold variation in IL-10 production between individuals; twin and family studies suggest that up to 75% of the variability is due to genetic factors [9]. Production is controlled at the transcription level [10] and some of the variability can be accounted for by two microsatellite polymorphisms (IL10G and IL10R) in the promoter region [11]. Eleven single nucleotide polymorphisms (SNPs) have also been described in the promoter region of which three are in the proximal 1.3 kb [–1082(G/A), –819(C/T), –592(C/A)] and seven in the distal 1.3–4 kb of which three [3575(T/A), 2849(G/A), 2763(C/A)] have equal allele frequency [12, 13]. In 26 normal Caucasian subjects the distal haplotype AA/GA was more frequent in those who produced less IL-10 [12]. In Afro-Caribbean lupus patients the frequency of the –2763A allele was less. No other associations with autoimmune rheumatic diseases are described with the distal SNPs. Of the proximal SNPs the –819 and –592 are in linkage disequilibrium [13]. Only three haplotypes are common in Caucasian subjects: GCC, ACC and ATA; GTA is more common in the Southern Chinese [14]. The GCC/GCC genotype is more common in those who produce higher IL-10 levels in whole blood cultures while the ATA/ATA genotype predominates in the lower IL-10 producers [12].

In this study we tested the hypothesis that the proximal genotypes linked to high IL-10 production will be more common, or that genotypes linked to low IL-10 production will be less common, in ISSc than in dSSc. Alternatively, genotypes linked to high IL-10 production are less frequent or genotypes with low IL-10 production are more frequent in dSSc.

Patients and methods

Patients

A total of 140 SSc patients were recruited, all of whom fulfilled the criteria of the American College of Rheumatology [15]. Disease subsets were defined as previously described [16]. All the patients were Caucasian, 51 having dSSc and 89 ISSc; 94 normal Caucasian adults from the West of Scotland served as controls.

Isolation of genomic DNA

Using the Nucleon BACC I kit (Scotlab, Coatbridge, UK) genomic DNA was isolated from 10 ml of EDTA-anti-coagulated blood. The genomic DNA was used as a template in the polymerase chain reaction (PCR).

Amplification refractory mutation system (ARMS) PCR

ARMS PCR was used to analyse the polymorphisms at positions –1082 and –819 in the 5' flanking region of the IL-10 gene as previously described [17].

The PCR was performed in a total volume of 10 μ l containing IX reaction buffer (ABgene, Epsom, UK), 8.5%

(w/v) sucrose, 5 μ M of generic primer, 5 μ M of allele specific primer, 1 μ M of each control primer (Table 1) and 0.25 units of Thermoprime^{Plus} DNA polymerase (ABgene). Between 25 and 100 ng of DNA was used. The following cycles were used to amplify the region of interest: 95°C for 15 s, 65°C for 50 s, and 72°C for 50 s with a final extension at 72°C for 5 min. Amplified PCR products were then analysed on a 2% agarose gel containing ethidium bromide.

Statistical analysis

Differences between groups were analysed on the statistical package, Minitab, using a χ^2 -test. Genotype distribution in the control population was compared with that observed in ISSc and dSSc. The number of copies of each haplotype (GCC, ATA, ACC) was also compared between these groups. Median values and interquartile ranges (IQR) were also assessed, with $P < 0.05$ taken as being significant.

Results

Demographic characteristics

There were no major demographic differences between the dSSc and ISSc patient populations. The ISSc group consisted of 79 females and 10 males with a median age of 58 yr (IQR: 50–66). The dSSc group comprised of 45 females and six males with a median age of 59 (IQR: 48–62).

Genotype distribution

There were no significant differences between patients with SSc (ISSc and dSSc) and the control population. However, fewer dSSc patients carried the GCC/GCC genotype (phenotypically characterized by high IL-10 production) compared with controls (controls vs dSSc; 29 vs 4%, $\chi^2 = 15.6$, 5 df, $P = 0.005$) and ISSc patients (ISSc vs dSSc; 22 vs 4%, $\chi^2 = 17.5$, 5 df, $P = 0.002$) (Table 2). No difference was observed between controls and ISSc patients.

IL-10 haplotype

No significant difference was observed in haplotype distribution between patients with SSc and the control population. However, the GCC haplotype was less frequent in the dSSc patients compared with controls (controls vs dSSc; 54 vs 36%, $\chi^2 = 11.2$, 2 df, $P = 0.001$). There was also a significant difference between dSSc and ISSc patients (dSSc vs ISSc; 36 vs 48%, $\chi^2 = 13.5$, 2 df, $P < 0.001$) (Table 3), but no difference between controls and ISSc patients.

TABLE 1. Primer sequences used for ARMS PCR

Primer	Product size (bp)	
–1082 Generic	5'-cagtccaactgagaatttgg-3'	
–1082 Primer G	5'-ctactaaggcttcttggag-3'	258
–1082 Primer A	5'-actactaaggcttcttggaa-3'	258
–819 Generic	5'-aggatgtgtccaggctcct-3'	
–819 Primer C	5'-ccctgtacaggtgatgaac-3'	233
–819 Primer T	5'-accctgtacaggtgatgaat-3'	233

TABLE 2. Genotype distribution between groups

	GCC/GCC	GCC/ACC	GCC/ATA	ACC/ACC	ACC/ATA	ATA/ATA
Controls (<i>n</i> = 94)	27 29%	18 19%	30 32%	8 8%	8 8%	3 3%
dSSc (<i>n</i> = 51)	2 4%	18 35%	15 29%	8 16%	6 12%	2 4%
lSSc (<i>n</i> = 89)	19 22%	18 20%	30 34%	3 3%	10 11%	9 10%

TABLE 3. Haplotype distribution between groups

	GCC	ACC	ATA	Totals
Controls (<i>n</i> = 94)	102 54%	42 22%	44 24%	188
dSSc (<i>n</i> = 51)	37 36%	40 39%	25 25%	102
lSSc (<i>n</i> = 89)	86 48%	34 19%	58 33%	178

Discussion

In this study we show that, as a group, SSc patients do not differ from controls in the distribution of IL-10 genotypes sought. Interestingly, dSSc patients are less likely to carry the genotype GCC/GCC, which is associated with higher IL-10 production. However, we are unable to comment whether the few patients with the GCC/GCC genotype had less severe disease, a different pattern of organ involvement or complications, as this data was not sought at study onset. In contrast, lSSc patients had a similar distribution of genotypes associated with high and low IL-10 production. The differences observed between dSSc and lSSc suggest that inheritance of IL-10 genotypes may be one of the many molecular events that determines the clinical phenotype.

IL-10 is a plausible candidate gene to study in the pathogenesis of SSc, not only because of its anti-inflammatory properties, but also because it protects against fibrosis. IL-10 reduces constitutive and TGF β 1-induced type 1 collagen mRNA expression in human lung fibroblasts. Furthermore, IL-10 reduces collagen and fibronectin production from fibroblasts [18]. Other candidate genes with a positive association in SSc include the C allele at codon 10 in the TGF β 1 gene (*n* = 152, odds ratio: 1.95), the angiotensin-converting enzyme D allele (*n* = 73, odds ratio: 3.4) and the endothelial nitric oxide synthetase gene 894T allele (*n* = 73, odds ratio: 1.9), but no correction was made for multiple testing in the latter study [19, 20]. Associations with MHC class II alleles are also reported [21] as is an association with the fibrillin-1 gene in Choctaw Indians [22].

The functional relevance of proximal SNPs in the 5' flanking region of the IL-10 gene is well defined at the transcriptional level [23]. The ATA haplotype results in lower transcription than the GCC or ACC haplotypes. In whole blood cultures the GCC/GCC genotype produces significantly more IL-10 than the ATA/ATA genotype. The SNPs reported in this study have been sought in other autoimmune rheumatic diseases. Patients

with juvenile chronic arthritis with more than four affected joints are more likely to carry the ATA haplotype (linked with less IL-10 production), as do those with more severe rheumatoid arthritis [23, 24]. The haplotypes linked to high IL-10 levels associate with some of the features of systemic lupus erythematosus [25]. GCC is also more frequent in patients with Sjögren's syndrome, whereas ACC is under-represented [26]. In non-rheumatic disorders, haplotypes linked to higher IL-10 production are more frequent in those with a better outcome after a renal allograft and less common in those with severe asthma [27, 28].

There are several limitations to our study. The patients studied were recruited from several centres rather than being part of an inception cohort and the data may therefore be skewed in favour of survivors. However, this is a limitation of any study in a relatively rare disorder in which large numbers are required. Ideally, we would also have liked to confirm the genotype and IL-10 production in SSc, but lack of resources did not allow us to collect serum or whole blood. As a genetic component has been shown to contribute to Raynaud's phenomenon, it may have been interesting to have a control population with primary Raynaud's. As this was not considered in the initial study design we did not obtain a history of Raynaud's from the controls studied. Another limitation of our study may be in the methods chosen for detection of PCR product, these were optimized in our laboratory in collaboration with the original investigators who described the method. To confirm our results, 20 random samples were analysed by the same investigator (A. C.) without prior knowledge of results; there were no differences. Resource limitations did not allow us to confirm the results by sequencing.

Our findings suggest that while the SNPs studied are not linked to the development of SSc they may influence the disease variant expressed. These findings need to be confirmed in a larger, preferably an inception, cohort. It would also be of interest to link clinical severity with genotype in those with dSSc.

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Conflict of interest

The authors have declared no conflicts of interest.

<i>Rheumatology</i>	Key Message
	This paper describes for the first time the study of the polymorphic IL-10 gene in patients with systemic sclerosis. Differences observed between subgroups of the disease may be clinically useful.

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