EXTENDED REPORT

Analysis of transforming growth factor β1 gene polymorphisms in patients with systemic sclerosis

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Objectives: To determine the distribution of transforming growth factor β1 (TGFβ1) genotypes at codon 10 (+869 polymorphism) and codon 25 (+915 polymorphism) in patients with scleroderma (SSc). Differences between diffuse and limited SSc (dSSc and lSSc) were also investigated.
Methods: Patients with lSSc (n=89) and dSSc (n=63) were compared with 147 controls. DNA was isolated from peripheral blood and polymorphisms at codons 10 (C/T) and 25 (G/C) of the TGFβ1 gene analysed by polymerase chain reaction and sequence specific oligonucleotide probing.
Results: Significantly more patients with SSc than controls carried allele C at codon 10 (controls *v* SSc, 38% *v* 48%, χ^2=8.2, 1df, p=0.004), OR=1.95 (95% CI 1.16 to 3.27). The difference remained when patients with SSc were split into those with limited or diffuse disease, (controls *v* dSSc, χ^2=5, 1df, p=0.02 and controls *v* lSSc, χ^2=6, 1df, p=0.013). The patients with SSc had significantly more subjects heterozygous at codon 10 (controls *v* SSc, χ^2=45, 1df, p<0.0001). Possession of allele C at codon 10 gave an OR=4.8 (95% CI 2.8 to 8.4). No difference in allele frequency was seen between patients with SSc and controls at codon 25. More patients with SSc than controls carried the GG genotype (controls *v* SSc, 80% *v* 88%, χ^2=7, 2df, p=0.027). Possession of allele G gave an OR=1.7 (95% CI 0.5 to 5.9). There was no difference between diffuse and limited disease at either codon.
Conclusions: These results suggest that patients with SSc are genetically predisposed to high TGFβ1 production. These polymorphisms do not, however, explain the difference in the clinical phenotypes of limited and diffuse SSc.

Systemic sclerosis (SSc) describes a group of disorders characterised by skin and visceral fibrosis, arteriolar myointimal proliferation, loss of the capillary bed, and chronic inflammatory cell infiltrate.[1] Clinically, two distinct patterns are recognised, diffuse (dSSc) and limited (lSSc). In dSSc both fibrotic and vascular changes are present, whereas in lSSc vascular changes predominate. Although overlaps do occur, each has a distinct clinical phenotype and outcome.

The fibrosis in SSc is thought to be initiated by cytokine/growth factors released from the inflammatory infiltrate.[2] Of these, transforming growth factor β1 (TGFβ1) is a known potent stimulus for extracellular matrix deposition and resorption.[2–5] The role of TGFβ1 in SSc has been extensively studied. In affected skin, particularly early in the course of the disease, there is marked overexpression of TGFβ1 and TGFβ2 but not TGFβ3.[6] In vitro cultures from SSc skin fibroblasts produced more glycosaminoglycans, collagen types I and II as well as fibronectin[7 8] in response to TGFβ1. SSc fibroblasts also express TGFβ1 mRNA, suggesting both paracrine and autocrine loops in SSc fibroblasts.[9] Mononuclear cells from bronchoalveolar lavage fluid from patients with SSc with lung fibrosis have increased TGFβ1 production.[10] In a murine SSc model based on graft versus host disease, fibrosis was inhibited by TGFβ1 neutralising antibodies.[11] Based on such findings, a fundamental role for TGFβ1 in SSc has been postulated.[2 3] It is, however, not clear why patients with SSc produce increased amounts of TGFβ1.

Seven TGFβ1 gene polymorphisms have been described, of which five have been confirmed in a subsequent study.[12 13] The two signal sequence polymorphisms at +869 and +915 are linked to disease outcomes.[12–17] The +869 polymorphism at codon 10 is a T→C substitution, resulting in a leucine→proline. The +915 polymorphism at codon 25 is a G→C substitution, resulting in arginine→proline. At codon 10, allele C is associated with higher TGFβ1 mRNA and protein levels.[14–16] In a study of a cohort of African Americans, hypertension was linked to codon 10 proline.[14] In a case-control study of Japanese postmenopausal women, codon 10 proline (TC or CC genotypes) was associated with higher bone mineral density and fewer vertebral fractures.[15] In a subsequent community study the CC genotype was associated with higher bone mineral density at the distal radius, while both normal and osteoporotic women carrying CC or TC genotypes had significantly higher serum levels of TGFβ1.[16]

At codon 25, the GG genotype results in more TGFβ1 production from stimulated lymphocytes than heterozygotes.[13] In a European study of 563 patients with a myocardial infarct the codon 25 genotypes of GC or CC were associated with an increased risk of subsequent myocardial infarction but not with the extent of the angiographic coronary atheromatous disease.[12] Those with a CC genotype were less likely to be hypertensive, a finding confirmed in a separate study.[12 17] The high producing codon 25 TGFβ1 genotypes, GG or GC, are more frequent in fibrotic lung disease requiring lung transplantation and in allograft fibrosis.[13] In a retrospective study of lung transplant recipients those homozygous for leucine (TT) at codon 10 and arginine (GG or GC) at codon 25 had a poorer outcome than all other TGFβ1 genotypes.[13]

We postulated that TGFβ1 alleles which increased TGFβ1 production at codon 10 (CC or TC) and the GG and GC genotypes at codon 25 would be significantly overrepresented in SSc. A secondary hypothesis was that differences in TGFβ1 genotypes may explain the two main clinical phenotypes of dSSc and lSSc.

Abbreviations: dSSc, diffuse systemic sclerosis; lSSc, limited systemic sclerosis; OR, odds ratio; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; SSc, systemic sclerosis; TGFβ1, transforming growth factor β1

Table 1 Primer and biotinylated probe sequences for detection of TGFβ1 gene polymorphisms at codon 10 and codon 25

Primer	Primer sequence	Annealing temperature (°C)
Sense	5-CTTCACCAGCTCCATGTCGATAG-3	60
Antisense	5-CTTCACCAGCTCCATGTCGATAG-3	60
Codon	Biotinylated TGF β1 probes	Stringency temperatures (°C)
10*C	5-GCTGCTGCCGCTGCTGC-3	58
10*T	5-GCTGCTGCTGCTGCTGCT-3	58
25*G	5-GCCTGGCCGGCCGGCCG-3	62
25*C	5-GCCTGGCCCGCCGGCCG-3	62

Table 2 Characteristics of controls and patients with diffuse (dSSc) and limited systemic sclerosis (lSSc)

	Male:female ratio	Median age	Interquartile range
Controls (n=147)	51:96	57	47-66
lSSc (n=89)	6:83	59	50-68
dSSc (n=63)	8:55	54	43-61

PATIENTS AND METHODS

Patients

A total of 152 patients were recruited. We had calculated that at 90% power to show a 25% difference we would require 61 patients in each of our study groups. Of the patients recruited, 63 had dSSc and 89 lSSc. All patients had SSc as defined by the American College of Rheumatology.[18] A normal group from the west of Scotland (n=147) served as controls.

DNA extraction and polymorphism analysis

Genomic DNA was isolated from 10 ml of peripheral blood collected in EDTA, using the Nucleon DNA extraction kit (Tepnel Life Sciences PLC, UK). DNA was amplified by polymerase chain reaction (PCR), and polymorphisms at codon 10 and codon 25 determined using sequence specific oligonucleotide probes for each allele as previously described.[19] Table 1 summarises the primers and biotinylated probe sequences with reaction conditions. Briefly 2 μl of PCR product was blotted onto Hybond N+ nitrocellulose membrane. Membranes were washed in denaturing solution (0.5 M NaOH/1.5 M NaCl) for five minutes and then neutralising solution (1.5 M NaCl/0.5 M Tris, pH 7.5) for one minute before being dried at 80°C for 15 minutes. DNA was immobilised using a crosslinking ultraviolet Stratalinker at 120 mJ.

Membranes were then washed for 30 minutes at 42.5°C in prehybridisation buffer (5×SSC: 0.75 M NaCl and 0.075 M sodium citrate/0.5% milk powder/0.1% N-laurylsarcosine and 0.02% sodium dodecyl sulphate (SDS)). Specific biotinylated probe was prepared in prehybridisation buffer (400 ng) and incubated overnight with membranes at 42.5°C. Membranes were washed twice at room temperature in 5×SSC/0.1% SDS for five minutes before being washed in 1×SSC/0.1% SDS for 30 minutes. Table 1 gives the temperatures for stringency washes with specific probes. Membranes were washed for one minute in 0.15 M NaCl/0.1 M Tris buffer, pH 7.5, before being incubated for 30 minutes at room temperature in the same buffer containing 0.5% milk powder. After blocking, membranes were incubated for 30 minutes at room temperature with streptavidin/horseradish peroxidase conjugate (Amersham) which was prepared in phosphate buffered saline (PBS)/0.1% Tween. Membranes were washed three times at room temperature for five minutes in PBS/0.1% Tween before a final 15 minute wash. Detection of probes bound to membrane was by chemiluminescence using an ECL Plus system (Amersham) and *x* ray film.

Statistical analysis

Differences between groups were analysed using χ^2 tests with $p<0.05$ taken as significant.

RESULTS

Demographic characteristics of groups

No major demographic differences between the controls, and patients with lSSc or dSSc were seen (table 2).

Codon 10 polymorphism

A significant increase in the frequency of allele C was found in all patients with SSc (diffuse and limited disease) compared with the control group (controls *v* SSc, 38% *v* 48%, $\chi^2=8.25$, 1df, p=0.004). When the patients with SSc were split into those with diffuse and limited disease, this increase in the frequency of allele C was still seen in comparison with controls (controls *v* dSSc, $\chi^2=5$, 1df, p=0.02 and controls *v* lSSc, $\chi^2=6$, 1df, p=0.013) (table 3).

When genotypes were examined, patients with SSc had significantly more heterozygous subjects than controls (controls *v* SSc, 37% *v* 74%, $\chi^2=45$, 1df, $p<0.0001$). Possession of allele C in the SSc group (TC/CC) gave an odds ratio (OR)=4.8 (95% CI 2.8 to 8.4). Similarly, patients with SSc split into groups with diffuse and limited disease had significantly more subjects heterozygous for TC than the control group (controls *v* dSSc, 37% *v* 81%, $\chi^2=35$, 2df, $p<0.0001$ and controls *v* lSSc, 37% *v* 70%, $\chi^2=26$, 2df, $p<0.0001$) (table 4).

There was no significant difference in either allele frequency or genotype distribution between patients with limited and diffuse disease (tables 3 and 4).

Table 3 Allele frequencies for TGFβ1 gene polymorphisms at codon 10 and codon 25 in controls (Ctrls) and patients with diffuse (dSSc) and limited systemic sclerosis (lSSc). Results are shown as No (%)

Allele	dSSc (n=63)	lSSc (n=89)	Ctrls (n=147)	p Values
Codon 10				
T	65 (52)	92 (52)	186 (63)	Ctrl *v* dSSc, p=0.02
C	61 (48)	86 (48)	108 (37)	Ctrl *v* lSSc, p=0.013
				dSSc *v* lSSc, NS
Codon 25				
G	117 (93)	162 (91)	261(89)	Ctrl *v* dSSc, NS
C	9 (7)	16 (9)	33 (11)	Ctrl *v* lSSc, NS
				dSSc *v* lSSc, NS

Table 4 Codon 10 polymorphism in controls (Ctrls) and patients with diffuse (dSSc) and limited systemic sclerosis (lSSc). Results are shown as No (%)

Genotypes	dSSc (n=63)	lSSc (n=89)	Ctrls (n=147)	p Values
Codon 10				
TT	7 (11)	15 (17)	66 (45)	Ctrl *v* dSSc, p<0.0001
TC	51 (81)	62 (70)	54 (37)	Ctrl *v* lSSc, p<0.0001
CC	5 (8)	12 (13)	27 (18)	dSSc *v* lSSc, NS
Codon 25				
GG	56 (89)	78 (88)	118 (80)	Ctrl *v* dSSc, NS
GC	5 (8)	6 (7)	25 (17)	Ctrl *v* lSSc, p=0.049
CC	2 (3)	5 (6)	4 (3)	dSSc *v* lSSc, NS

Codon 25 polymorphism

There was no significant difference in the distribution of the G and C allele at codon 25 between patients with SSc and controls. Similarly, no difference in allele frequency was seen when patients with SSc were split into those with limited and diffuse disease (table 3).

When genotypes were examined, GG showed a trend to increased frequency in SSc compared with controls (controls *v* SSc, 80% *v* 88%, $\chi^2=7$, 2df, p=0.027). Possession of allele G in the patients with SSc (GG/GC) gave an OR=1.7 (95% CI 0.5 to 5.9). When patients with diffuse or limited SSc were considered separately both groups had a higher frequency of the GG genotype, but this was only significant in lSSc (controls *v* lSSc; 80% *v* 88%, $\chi^2=6$, 2df, p=0.049) (table 4).

There was no difference in either allele frequency or genotype distribution between the patients with limited and diffuse disease (tables 3 and 4).

DISCUSSION

The results of this study show that the TC genotype is significantly more common in SSc. The presence of proline rather than leucine in the hydrophobic region of the signal sequence is thought to alter protein export across the endoplasmic reticulum.[20] The presence of proline because of its cyclic structure will alter the α helical portions of the signal peptide backbone, whereas leucine owing to its aliphatic side chain will favour the formation of α helices. This is thought to affect transfer of protein through the endoplasmic reticulum. The normal variation in TGFβ levels has also been attributed to two other polymorphisms G→A at position –800 bp and C->T at position 509 bp, which are in linkage disequilibrium.[21] We elected not to examine these alleles because no disease associations have yet been reported. Variations in serum TGFβ1 levels have been reported in healthy controls, which have been attributed to genetic differences, the difference between CC and TT being approximately 17%.[15] Owing to the limitations of serum sample availability and resource, we were unable to measure serum levels and link this to genotype. There was no difference in allele frequency at codon 10 between dSSc and lSSc. Although the GG genotype at codon 25 was more common in patients with SSc than in controls, this was not seen when the patients were split into those with limited and diffuse disease. No other associations were noted with the codon 25 polymorphism.

In a previous study of 19 North American Choctaw Indians, in whom there is a high prevalence of SSc, an association with codon 10 allele C was noted, but this was not statistically significant after correction for multiple testing.[22] A type I or random error was minimised in our study by a prior power calculation in which we made the assumption that there was at least a 25% difference between those with and without the C allele. The differences at codon 10 remained between all patients with SSc and controls after correcting for testing at two polymorphisms.

The OR for patients with SSc carrying a C allele at codon 10 (TC or CC) was 4.8 (95% CI 2.8 to 8.4). Other polymorphisms linked to SSc susceptibility include the major histocompatibility complex. Despite extensive study, no definite pattern has emerged. In the largest single centre study done on 206 patients with SSc by Steen *et al*,[23] a weak association with HLA-DR5 was reported in patients with dSSc, and HLA-DR1 in patients with lSSc. An association between HLA-DR52a and pulmonary fibrosis has been reported in those with diffuse disease.[24] A chromosome 15q haplotype, which includes the fibrillin gene, has also been reported.[25] Other reported associations include SSc pulmonary fibrosis with some restriction fragment polymorphisms of the fibronectin gene.[26]

In this study all patients recruited were required to have either dSSc or lSSc as defined by the American College of Rheumatology. Of the patients gathered, 122 were from rheumatologists and the remainder were obtained by an appeal in a national patient SSc newsletter. Confirmatory diagnosis in these patients was sought from their rheumatologists or primary care doctor. Although we consider we can be certain of the diagnosis and type of SSc, no comment can be made on the overall disease severity, pattern of organ involvement, or complications in relation to the alleles studied. A much larger cohort with more accurate characterisation of disease features would be required. A further limitation of this study is that we cannot exclude the possibility of a survivor bias. A recent cohort study of dSSc reported that those with severe disease at onset had a higher early mortality.[27] Possibly, our study is biased towards those with milder disease.

The method of detection of PCR products chosen for study had been optimised and previously described.[13] To confirm our results a proportion of the samples was sequenced without prior knowledge of results by an independent commercial company (results not shown). No differences were noted.

Confirmation of our initial findings is required in a larger cohort. It would also be of interest to establish disease features with this polymorphism. If our findings are confirmed this may be one explanation for the increased TGFβ1 levels seen in SSc. It would also be of interest to examine associations with promoter region polymorphisms, which have been associated with increased serum levels.[21]

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