Review
Genetic factors in systemic sclerosis

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Abstract

A number of genetic loci have been identified that appear to be associated with systemic sclerosis (SSc; scleroderma). There is mounting evidence suggesting that these genetic associations may in fact be associated with distinct phenotypes in SSc based on autoantibody pattern rather than with SSc as a single disease entity. This may ultimately have implications for approaches to therapy as well as responses to therapy. The most promising candidate genes are those involved in pathways that lead to the vascular damage and fibrosis that are the hallmarks of this disease. There is uncertainty, however, regarding the nature of the key pathological mechanisms that link these two disease processes. Recent studies have focused on Fli1 (friend leukaemia integration 1), a transcription factor that is found in immune cells, fibroblasts, and endothelial cells that regulates collagen gene function and angiogenesis. Fli1 is dysregulated in SSc skin and dermal blood vessels, and appears to play a pathological role in SSc skin fibrosis and vessel degeneration. Whether this dysregulation is due to genetic polymorphisms in the Fli1 pathway or to epigenetic mechanisms is not clear.

Introduction

It is the generally accepted dogma that autoimmune disease requires an external trigger to act upon a genetically susceptible host. Identifying the underlying nature of this susceptibility represents a considerable challenge that can be attempted using a variety of approaches, including familial, twin and genetic studies. This review begins by discussing some of the available evidence for the existence of a genetic component in systemic sclerosis (SSc; scleroderma). It then focuses on studies conducted to determine the role played by a particular gene product, Fli1 (friend leukaemia integration 1), whose dysregulation appears to contribute to the complex pathogenesis of SSc in a number of ways.

Evidence for a genetic component to systemic sclerosis
Familial studies

A positive family history is the strongest risk factor thus far identified for SSc. A large US cohort-based study [1] found that although the absolute risk in individual family members was quite low (<1%), in a small but significant proportion of families (1.6%) more than one first-degree relative was affected, giving a familial relative risk of 13. A similar study in Australia [2] reported a recurrence rate in families of approximately 1%, which is in relative agreement with the US estimate of 1.6%.

In the current Scleroderma Family Registry of 693 index cases, in 16 families more than one person is affected; there are five sibling pairs and 11 parent-offspring pairs [3]. Unfortunately, the rarity of SSc precludes the generation of meaningful genetic information from family-based linkage studies, which have proved to be helpful in identifying genetic associations in other more common autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis.

In general, studies of twins are considered the 'gold standard' for determining whether a disease is due to shared genetic predisposition or to shared environmental factors, whereby a higher concordance rate for a disease is expected in monozygotic than in dizygotic twins. In the only published SSc twin study to date [4] the concordance rate was low and no higher in monozygotic twins than in dizygotic twins. However, the incidence of antinuclear antibody positivity was higher in monozygotic twins than in dizygotic twins (90% versus 40%), suggesting at least a greater tendency toward disease in the former.

ACA = anticentromere; ATA = antitopoisomerase; CTGF = connective tissue growth factor; Fli1 = friend leukaemia integration 1; HLA = human leucocyte antigen; PTPN22 = protein tyrosine phosphatase non-receptor type 22; SNP = single nucleotide polymorphism; SSc = systemic sclerosis; TGF = transforming growth factor.

Genome-wide studies

To date only one genome-wide association study in scleroderma has been reported. The Choctaw study [5] localized chromosome regions associated with susceptibility to SSc in a relatively isolated and homogeneous population of Choctaw Indians with a high prevalence of SSc. The affected individuals all had diffuse disease with pulmonary fibrosis, with over 80% being positive for Scl70. The study identified a total of 17 chromosome regions that were associated with SSc, including some that overlapped with regions identified in genome-wide studies of systemic lupus erythematosus and rheumatoid arthritis (Table 1; for reviews see the reports by Lee and Nath [6] and Etzel and coworkers [7]). One of the regions identified in this way is the human leucocyte antigen (HLA) region on 6p. The identity of other genes within these regions is currently unknown. It will be interesting to see respective data from a genome-wide scan in outbred non-Choctaw populations that is currently being undertaken in a study comparing approximately 5,000 SSc patients with 15,000 control individuals.

What are the candidate genes in systemic sclerosis?

The choice of which candidate genes to study can be daunting, considering the approximately 26,000 known genes. There are several approaches to this dilemma. The first is to choose genes in the regions that have been identified by the genome-wide scan [5]. The second is to study genes that have been identified in other autoimmune diseases and that appear to predispose to immune dysregulation. The third is to choose genes that play a key role in the aberrant pathways for a specific disease. As outlined below, all three approaches have been used in the study of scleroderma.

From a technical point of view, one of the methods used to identify candidate genes associated with a disease involves single nucleotide polymorphism (SNP) analysis. An SNP defines a genetic locus in which two or more alleles have gene frequencies greater than 1% in the population. SNP maps can help to identify multiple genes associated with complex diseases, which is difficult to achieve with conventional gene-hunting methods, because a single altered gene may make only a small contribution to the disease. The SNPs of greatest interest are nonsynonymous SNPs, namely those in which a single nucleotide change will yield a change in the encoded protein.

A number of interesting polymorphisms or gene variations have been associated with SSc (Table 2; for review, see the report by Assassi and Tan [8]), some of which are discussed in more detail below. However, one should bear in mind that SSc is a heterogeneous disease with multiple subtypes and that associations with particular polymorphisms may be based on the ethnic background of the populations studied. For example, although one report suggests an association of

Table 1

Gene regions significantly associated with systemic sclerosis in the Choctaw Indian population

Region on chromosome	Marker	P value
1p32-31	D1S255	0.0068
1p21.2[a]	D1S206	<0.0001
1q42.3[a]	D1S2800	0.0012
5q33.2[a]	D5S410	<0.0001
6p22.3[a]	D6S422	0.0368
6q23-27[a]	D6S264	0.0176
7p12-11[a]	D7S510	0.0017
7q35	D7S661	0.005
8q24.12	D8S514	0.0015
14q21	D14S63	0.0173
15q21.1[a]	D15S978	0.0189
19p13.2	D19S221	0.0016
19q13.2[a]	D19S220	0.0175
20q12	D20S107	0.0095
22q13.1	D22S423	0.0005
Xp11.4	DXS1068	0.0026
Xq21-23	DXS8055	0.0082

[a]Regions that are common to systemic lupus erythematosus and/or rheumatoid arthritis.

endothelial nitric oxide synthase with SSc [9], other groups found no such association [10,11]. In general, disease associations with particular SNP polymorphisms are usually not considered 'true' until they have been confirmed in an independent cohort.

Similarly, there are conflicting reports on the association of *SPARC* (secreted protein, acidic and rich in cysteine) polymorphisms with SSc [12,13]. This lack of confirmation may be due to the fact that Zhou and coworkers [12] studied the Choctaw Indians, a relatively inbred group with homogeneous disease expression, whereas Lagan and coworkers [13] studied a more heterogeneous scleroderma cohort. This association must be confirmed (or rejected) in a scleroderma cohort with diffuse disease and antitopoisomerase antibodies (ATA). Until then, this association must be considered unverified.

PTPN22 R620W polymorphism

The *PTPN22* gene encodes the lymphoid-specific protein tyrosine phosphatase nonreceptor type 22, which resides on on the short arm of chromosome 1 (area 1p13). The presence of the minor allele R620W results in the substitution of tryptophan for arginine at the binding site of the protein, which disrupts its ability to bind to protein kinase,

Table 2

Candidate gene polymorphisms associated with systemic sclerosis

Encoded protein (gene location)	Patient (*n*)	Control (*n*)	Association
TNF promoter (6p21.3)	214	354	-1031 C allele and -863 A allele strongly associated with ACA-positive SSc [37]
TNF-α (6p21.3)	114	170	-238 and +489 A alleles are weakly associated with SSc [38]
TNF-RII (chromosome 9)	114	170	TNF-RII -196 polymorphism is not associated with SSc [38]
MCP-1 promoter (17q11.2)	18	139	-2518 G/G phenotype is associated with SSc [39]
IL-1A (2q14)	60	70	IL-1A gene polymorphism is associated with SSc [40]
IL-1A (2q14)	46	150	IL-1A -889T allele is associated with SSc [41]
eNOS (7q36)	73	112	eNOS is associated with SSc [9]
eNOS (7q36)	77	49	eNOS G894T is not associated with SSc [10]
eNOS (7q36)	164	184	eNOS G894T is not associated with SSc [11]
SPARC (5q31.3-32)	20	75	SPARC polymorphisms are associated with SSc [12]
SPARC (5q31.3-32)	121	200	SPARC polymorphisms are not associated with SSc [13]

ACA, anticentromere antibody; eNOS, endothelial nitric oxide synthase; IL, interleukin; MCP, monocyte chemoattractant protein; SPARC, secreted protein, acidic and rich in cysteine; TNF, tumour necrosis factor; TNF-RII, TNF receptor II.

thereby preventing its normal metabolism [14]. This increases PTPN22 protein activity and, as a result, the threshold required for T-cell receptor signalling in the thymus. It is speculated that the mutation might induce autoimmunity by preventing the deletion of autoreactive T cells, resulting in insufficient activity in regulatory T cells.

A positive association of the *PTPN22* R620W polymorphism has been identified for patients with SSc, and more specifically for the subgroup of SSc patients who are ATA positive and those who are anticentromere antibody (ACA) positive [15]. This study evaluated 1,120 SSc patients and 816 control individuals. The odds ratio in the ATA-positive patients compared with control individuals was 2.36 (95% confidence interval 1.5 to 3.6), and the odds ratio in the ACA-positive patients was 1.71 (95% confidence interval 1.1 to 2.6). Two other studies, namely those by Balada [16] and Wipff [17] and colleagues, failed to find an association with this polymorphism, but the number of cases and controls in these studies was quite small (the Balada study included only 54 cases and 55 controls, whereas the Wipff study included 121 cases and 103 controls). Therefore, the negative findings in these latter two studies are probably due to their being underpowered to detect an association with an odds ratio of this magnitude.

Positive associations have been found for a number of other immune diseases, including rheumatoid arthritis and systemic lupus erythematosus (Table 3). The *PTPN22* R620W poly-morphism is not uniformly associated with autoimmune diseases, however. There are some autoimmune diseases for which no association with this particular polymorphism has been found (Table 3; for review, see the report by Gregersen and Batliwalla [18]).

Table 3

***PTPN22* R620W polymorphism and links with immune diseases**

Association identified	No association
Scleroderma: ATA positive and ACA positive	Multiple sclerosis
RA and JRA	Coeliac disease
SLE	Primary sclerosis cholangitis
Type I diabetes mellitus	Crohn's disease
Grave's and Addison's	Psoriasis
Psoriatic arthritis	Ankylosing spondylitis
Wegener's granulomatosis	Primary Sjögren's syndrome

This area of research is reviewed by Gregersen and Batliwalla [18]. ACA, anticentromere antibody; ATA, antitopoisomerase antibody; JRA, juvenile rheumatoid arthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

AIF1 polymorphism

This polymorphism is located in the class III region, close to the tumour necrosis factor gene cluster. The encoded protein, allograft inflammatory factor 1, is detected in vasculopathic vessels, for example after balloon angioplasty. It is inducible in vascular smooth muscle cell cultures by proinflammatory cytokines and promotes activation and proliferation of vascular smooth muscles cells [19,20]. The gene is upregulated in skin biopsies and peripheral blood cells of scleroderma patients as compared with those of matched control individuals in gene expression studies [21-23].

A nonsynonymous SNP (*AIF-1* gene SNP rs2269475) that results in a tryptophan to arginine amino acid substitution is found at a higher frequency in patients with SSc than in control individuals [24]. To date, it is not clear whether the amino acid substitution affects the function of the protein, and therefore the functional significance of this change is unknown.

HLA gene region polymorphisms
The HLA gene region is an extreme example of genetic polymorphism. We recently completed a study of 1,100 patients in which we looked at several HLA class II associations and were able to verify the findings of multiple previous studies. Grouped by race or ethnicity, none of the HLA associations were consistent in all groups. However, grouped according to autoantibody status [25], for example ATA, ACA, or RNA polymerase, the findings were consistent between different ethnic groups. It therefore seems likely that the gene associations may in fact be associated with sub-phenotypes, particularly as defined by autoantibody expression, rather than with SSc as a single disease entity.

An important area of future research will be to determine whether these sub-phenotypes also predict response to therapy.

Gene profiling in systemic sclerosis
In general, the molecular and cellular mechanisms that maintain proper collagen homeostasis in healthy human skin and are responsible for the dysregulated collagen synthesis in SSc remain unknown. In an attempt to identify the genes that are involved in this process, gene profiling studies have been undertaken; gene profiling is a useful technique for comparing gene expression between normal and diseased cells. Gene profiling of SSc skin biopsies revealed robust signatures of disease, identifying changes in expression of about 1,800 genes that distinguish normal skin from SSc skin at a significant level [22]. Alterations in transforming growth factor-β and Wnt pathways, extracellular matrix proteins and the CCN family were prominent. However, the changes identified are not fully reflected in the profiles of explanted fibroblasts. This observation suggests that studies conducted in biopsy material are likely to prove more useful in the complex disease pattern of SSc than cultured fibroblasts.

In addition to gene polymorphisms, epigenetic mechanisms such as the degree of methylation or copy number variations can change gene expression and result in a pathological state. Such may be the case with Fli1.

The role of Fli1 in the pathogenesis of systemic sclerosis
Fli1 expression in fibroblasts
Fli1 belongs to the family of Ets transcription factors that were recently shown to be dysregulated in many immune diseases [26]. Until relatively recently, Fli1 was considered to

Figure 1

% Fli-1 positive fibroblasts
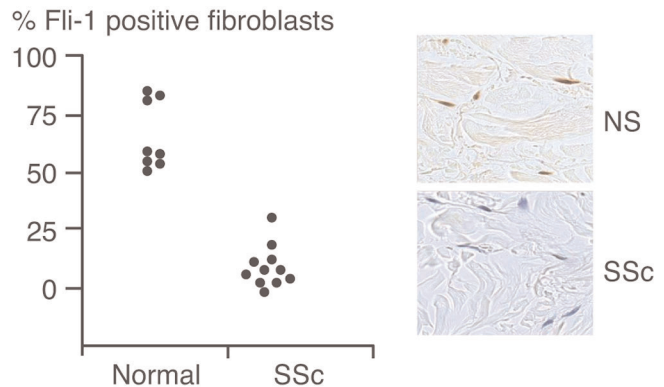
66 ± 14% vs 8 ± 8%, P<0.001

Fli1 is absent in the majority of fibroblasts in SSc skin. Fli1, friend leukaemia integration 1; NS, normal skin; SSc, systemic sclerosis. Reprinted from Kubo *et al. Am J Pathol* 2003, **163:**571-581 with permission from the American Society for Investigative Pathology [27].

be merely an immune cell transcription factor and was not known to be expressed in fibroblasts. However, the finding that Fli1 expression levels in cultured human and mouse fibroblasts are inversely correlated with collagen type I expression levels suggested that it may be a repressor of collagen genes. Evidence for a role for Fli1 in regulating collagen synthesis in cultured dermal fibroblasts and in human skin *in vivo* was recently provided. Immunostaining for Fli1 in normal skin biopsies revealed that although 70% of fibroblasts were positive for Fli1, there was little evidence of Fli1-positive fibroblasts in SSc skin biopsies (Figure 1). The absence of Fli1 correlated with enhanced collagen synthesis in SSc skin. Fli1 was shown to be required to maintain the repressed state of the fibrillar collagen genes [27,28]. A recent study suggested that epigenetic mechanisms may be responsible for downregulation of Fli1 in SSc skin *in vivo* [29].

In order to investigate how Fli1 maintains this repressed state, the effects of Fli1 expression on regulation of genes that were differentially expressed in SSc biopsies but not in normal skin were examined [30]. This study showed that Fli1 directly regulates the expression of connective tissue growth factor (CTGF), also known as CCN2. Downregulation of Fli1 upregulated expression of CTGF mRNA, which mimics to some extent the profibrogenic effects of transforming growth factor (TGF)-β. This is an important finding, because there is little detectable TGF-β during fibrosis in SSc, other than during the very early stage.

In addition to upregulation of CTGF, blocking Fli1 also affected the expression of some other genes in the biopsy. The expression of collagen mRNA was increased, as well as that of *PLOD2*, which is known to be very important for

collagen cross-linking [31]. As well as upregulating certain genes in SSc biopsies, blocking Fli1 resulted in the downregulation of others; one of these was the gene encoding decorin, which also probably contributes to fibrillar genesis.

Although the results from the *in vitro* studies on human fibroblasts strongly suggested that reduced expression of Fli1 had a profibrotic effect, this must also be demonstrated *in vivo*. The Fli1$^{-/-}$ genotype is lethal, with mice dying from cranial or spinal haemorrhages at day 11.5 [32]. Therefore, a heterozygous model was initially used, with a 50% reduction in Fli1 levels, which is viable and exhibits absolutely no phenotype. Levels of interstitial collagen mRNA were only slightly elevated in Fli1$^{+/-}$ mice; however, it could be demonstrated that they produced elevated levels of acetic acid extractable collagen and of type I collagen. Importantly, an increase in fibril diameter by electron microscopy could also be demonstrated, which is also found in SSc patients. Homozygous Fli1 mice carrying a *Fli1* gene that lacks a carboxyl-terminal domain (Fli1$^{\Delta CTA}$) were recently generated. Fibroblasts from these mice maintain the activated phenotype when cultured; these should prove useful in further elucidating the role played by Fli1 in pathogenesis.

In summary, the recent observations provide valuable support for the role of dysregulation of Fli1 in contributing to the development of fibrosis in SSc. What is not yet clear is what factors (genetic, environmental, or immunological) are responsible for downregulating Fli1 in SSc.

Fli1 expression in the vascular compartment and its potential role in vessel degradation

As well as being expressed in healthy fibroblasts, Fli1 is highly expressed in endothelial cells, and probably in pericytes and other cells around blood vessels. As well as being downregulated in affected SSc skin, Fli1 is also down-regulated in areas of uninvolved skin from SSc patients (Figure 2).

Staining of microvessels in the homozygous Fli1$^{\Delta CTA}$ mice compared with Fli1$^{+/+}$ mice revealed downregulation of a number of proteins that are known to be involved in endothelial-mural interactions. These included smooth muscle actin, desmin and platelet-derived growth factor receptor β. Similar features were observed in human biopsies. In healthy skin smooth muscle actin positive cells around the vessels may be clearly observed near the epidermis and in deep dermis. In SSc uninvolved skin we see malformed vessels that are still partially covered by pericytes, and in SSc involved skin we see very few vessels near the epidermis and none in deep dermis (Figure 3).

A possible explanation for why vessels degenerate in SSc relates to angiogenesis, because angiogenesis involves a number of sequential steps, including the following: detach-

Figure 2
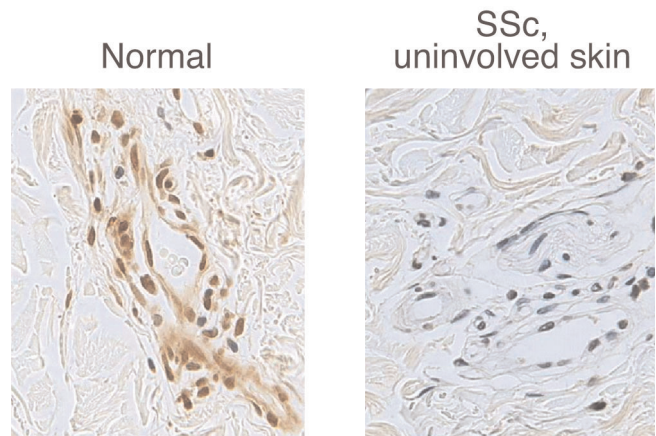
Fli1 is downregulated in microvessels in SSc skin. SSc, systemic sclerosis. Reprinted from Kubo *et al. Am J Pathol* 2003, **163**:571-581 with permission from the American Society for Investigative Pathology [27].

Figure 3
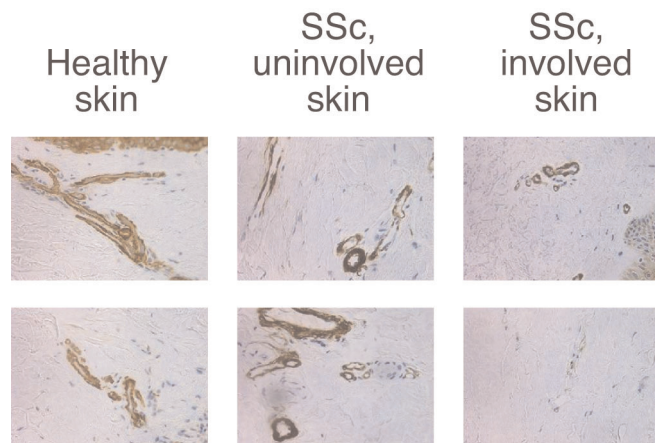
Comparison of α-SMA staining in healthy and SSc skin. SMA, smooth muscle actin; SSc, systemic sclerosis.

ment of pre-existing pericytes or vascular destabilization; extracellular matrix degradation by endothelial proteases; migration and proliferation of endothelial cells; tube formation by endothelial cells; and, finally, reattachment of pericytes for vascular stabilization [33,34]. In SSc skin it appears that the vessels undergo proliferation when angiogenesis begins, but they experience problems with reattachment of pericytes, vessel maturation and stabilization. Using a three-dimensional co-culture model to study the effect of Fli1 on dermal microvascular endothelial cells covered by human collagen and fibroblasts, we found that reducing Fli1 levels had a proangiogenic effect, with increased tube formation, cell migration and apoptosis in the collagen matrix [35]. These

findings suggest that in SSc there may be a problem with vessel stability or maturation, or some kind of interaction between endothelial cells and pericytes. Work in cancer has shown that if pericytes are not present, then such vessels do not survive [36].

Conclusion

Understanding of the genetic factors involved in SSc has increased considerably in recent years. There is doubtless a role for genes, in terms of either susceptibility or influencing the phenotypic expression of the disease. On a broad level, genetic studies are proving valuable in identifying genomic regions with possible associations with SSc and, at the same time, emphasizing the complexity of this disease, which appears to represent a collection of phenotypes rather than a single disease entity.

It is likely, based on results from PTPN22 studies and from HLA studies, that there will be 'general' autoimmune disease genes (such as *PTPN22*) and scleroderma specific genes (like the HLA region associations). This area is undergoing rapid change and is expected to provide new insights into pathogenesis as well as therapeutic approaches.

The findings of gene profiling investigations are beginning to provide us with possible mechanisms to explain, at least in part, some of the complex pathogenesis that is involved in SSc, with downregulation of Fli1 expression appearing to play a central role. However, there remains much to learn, including identification of the trigger(s) that result in the development of this complex disease.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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