

tive family history being the strongest relative risk for disease². In twin studies, the concordance rate for the clinical phenotype was low, whereas the concordance rate for anti-nuclear antibodies and SSc fibroblast phenotype of gene expression was significantly higher in monozygotic twins than in dizygotic twins^{3,4}.

The tight-skin 1 (*Tsk1/+*) mouse develops a thickened and abnormally structured dermis and is a model widely used for studying SSc. The *Tsk1/+* phenotype is the result of a partial in-frame duplication of the fibrillin-1 gene (*Fbn1*). This duplication results in a larger than normal protein. Also, autoantibodies directed against the proline-rich fibrillin-1 region have been found in the *Tsk1* mouse⁵.

Fibrillin-1 is a modular glycoprotein encoded by the large *FBNI* gene (230 kb). The *FBNI* gene maps to the 15q21.1 region and contains 65 coding exons. Fibrillin-1 is a major constituent of microfibrils in the extracellular matrix and is closely similar to latent transforming growth factor- β (TGF- β) binding proteins. These proteins bind to the small latent TGF- β complex and sequester it to the extracellular matrix⁶.

Genetic studies have been carried out in a Native American population (Choctaw Indians) with a high prevalence of the homogenous diffuse cutaneous SSc disease subtype. These studies detected an *FBNI*-containing 2-cM haplotype on chromosome 15q associated with SSc using microsatellite markers⁷. An *FBNI* single-nucleotide polymorphism (SNP) study revealed that some *FBNI* haplotypes were associated with SSc in both Native American and Japanese patients with limited cutaneous SSc⁸. A negative association with another SNP of the 5'-UTR region was found in a Japanese population⁹. Metabolic labeling studies suggested that fibrillin-1 synthesized by human SSc fibroblasts may be unstable¹⁰. These various findings strongly suggest that defects in fibrillin-1 may play an important role in the pathogenesis of SSc. We investigated the links between *FBNI* gene polymorphic markers and SSc in large populations of European Caucasian patients using a candidate gene approach in a case-control study.

MATERIALS AND METHODS

Patients. We included 399 consecutive unrelated subjects for the initial part of the study, comprising 243 French SSc patients classified for cutaneous subtypes according to LeRoy, *et al*¹¹ and 156 healthy controls matched for age and sex. Exclusion criteria were presence of Marfan's syndrome and congenital contractural arachnodactyly (Beals syndrome), which are associated with *fibrillin-1* gene mutation. All 399 subjects were of French Caucasian origin, defined by all 4 grandparents being French Caucasian, and were recruited at 3 rheumatology and 2 internal medicine departments. The Ethics Committee of Cochin Hospital approved the study and all patients gave written informed consent for all procedures.

We also investigated another cohort of European Caucasian patients with SSc to try to replicate our initial results; this cohort from Italy, recruited from 3 centers, had 266 patients with SSc and 153 matched controls.

Using newly available HapMap data (www.hapmap.org), we genotyped the French Caucasian cohort enlarged to 362 SSc patients (199 from the initial cohort and 163 new patients) and 162 controls for 5 tagging single

nucleotide polymorphisms (tagSNP) that account for the common genetic diversity of the *FBNI* gene. New patients and controls of the enlarged cohort were included with the same clinical criteria, from the same medical departments, and were of Caucasian origin as defined.

Clinical data were collected for age, sex, disease duration (date of first non-Raynaud symptom), and cutaneous SSc subtype¹¹. Lung involvement was assessed as follows: pulmonary fibrosis was investigated by computerized tomography scan, and a restrictive syndrome was defined as a forced vital capacity < 75% of the predicted value; vascular involvement was considered pathological if pulmonary arterial pressure measured by right catheterization in suspected patients was > 25 mm Hg at rest. Immunological tests were carried out for anticentromere antibodies (immunofluorescence on HEp-2 cells) and antitopoisomerase I (counter-immunoelectrophoresis).

Microsatellite genotyping. We tested 5 microsatellite markers: one microsatellite (D15S1028) mapping to the 5' upstream region of *FBNI* gene, 2 intragenic markers (MTS2 and MTS3), and 2 markers (D15S123 and D15S143) mapping to the 3' region (Figure 1A). Microsatellites were chosen for their heterozygosity and their location near or within the *FBNI* gene (Table 1). Markers were amplified by fluorescent polymerase chain reaction and genotyped on an ABI Prism-3100 (Applied Biosystems, Courtabouef, France) and analyzed with Genotyper software.

SNP genotyping. We also investigated the synonymous SNP (intron C, +49 C to T) that had been reported to be associated with SSc in Choctaw and Japanese populations⁸. Intron C maps to the 5' UTR of the *FBNI* gene (Figure 1). We used cold primers for amplifying intron C, as described⁸. The sequencing product was run on an ABI Prism-3100, and analyzed with Sequencing Analysis software.

The *FBNI* tagSNP tested in the second part of the study were selected using software from SeattleSNPs, Program for Genomic Applications (<http://pga.gs.washington.edu/VG2.html>) to provide the maximum amount of information with the smallest number of SNP to type in the *FBNI* gene according to HapMap data. Only 5 tagSNP were needed to account for 98% of the *FBNI* HapMap SNPs: rs7177445 (SNP1), rs1042078 (SNP2), rs682737 (SNP3), rs2289136 (SNP4), and rs11635140 (SNP5) (Figure 2). The tagSNP were genotyped using the TaqMan 5'-exonuclease allelic discrimination assay (Applied Biosystems).

Statistic analysis. Hardy-Weinberg equilibrium was tested for all polymorphisms assessed in each cohort using Arlequin software (L. Excoffier; [http://anthro-unige.ch/arlequin](http://anthro.unige.ch/arlequin)). This software was used to evaluate linkage disequilibrium (LD) between pairs of markers assessed using a likelihood ratio test. Haplotype block structure was defined using HaploView based on Gabriel block definition. We compared the allelic frequencies of markers between SSc patients and controls using Cocophase software (F. Dudbridge; www.rfcgr.mrc.ac.uk/fdudbrid/software). P values less than 0.05 were considered significant after correcting for the number of alleles with a frequency higher than 5% (non-rare allele) and the number of the assessed tests (Bonferroni correction). Results regarding comparison of frequencies were confirmed each time using Fisher's exact test (Statxact, Cytel Corp., Cambridge, MA, USA). *FBNI* haplotypes were constructed taking into account rare and non-rare alleles and using the maximum likelihood procedure implemented in Phase 2.0 software (M. Stephens, P. Donnelly, University of Chicago; www.stat.washington.edu/stephens/software.html).

Power calculations are driven through an asymptotic non-central chi-square approach as described¹². A sample of 362 SSc cases and 162 controls provides a power of 80.9% to detect an association between SSc and the minor allele of rs11635140 based on the allele frequency previously observed in the HapMap CEU population (NCBI database) with an OR of 1.5 at 5% significance level.

RESULTS

Population characteristics. We included 243 French patients with SSc in the initial French cohort, 209 women (86%),

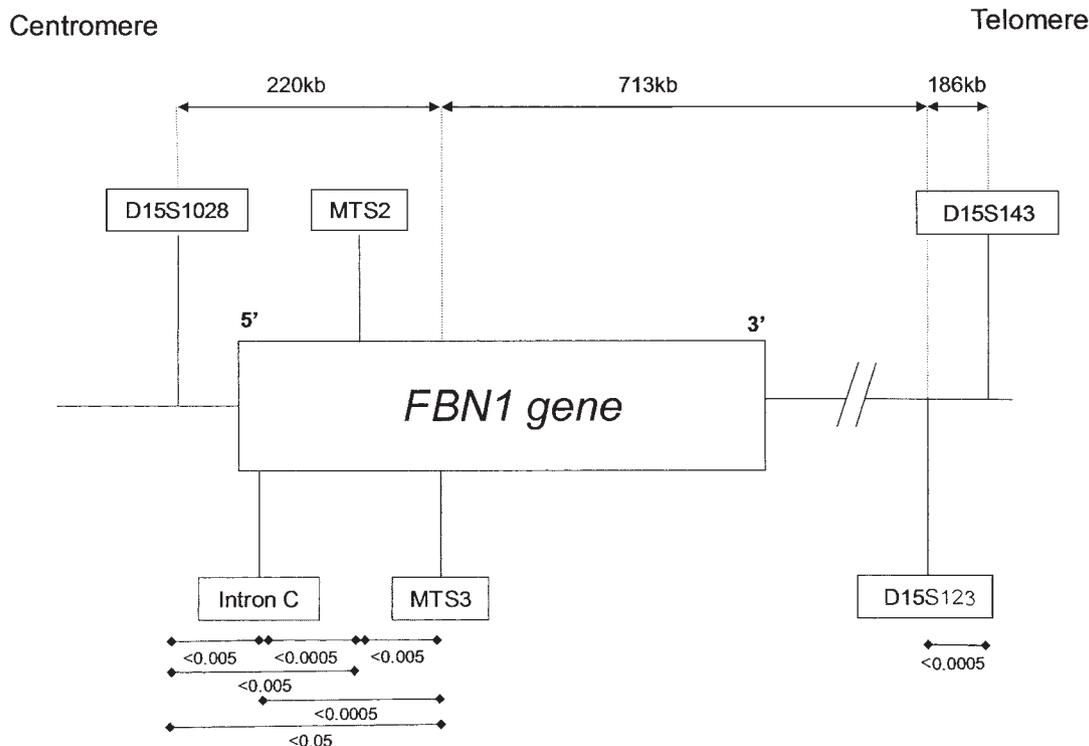


Figure 1. Map of the 5 microsatellite markers and the intron C SNP in the *FBNI* gene region. Allelic association analysis revealed 2 sets of markers in close linkage disequilibrium: the first (220 kb) contained the 4 markers D15S1028, intron C, MTS2, and MTS3 in the 5' region of the *FBNI* gene; the second (186 kb) contained the 2 markers D15S123 and D15S143 in the 3' region.

Table 1. Characteristics of the *FBNI* gene polymorphic markers tested.

	Sequencing Primers 5' to 3'	Nucleotide Location	Location*	Heterozygosity
D15S1028	TGT CCT GAA ATT CCC AAC GAA CTG TGC TCT GTG CTC	46,784,274 (chr 15)	5'	0.83
Intron C	GGC GTC AGA TGT TCG GGG GGC ACA GAG ACA AAT CCC CGA GG	46,724,785 (chr 15)	5' UTR	0.35
MTS2	GTA GTT GTT ATC TTG CAG ACT GCC CTC TAG GAC TCT AAG A	46,622,416 (chr 15)	Intron 5	0.75
MTS3	CCC TGG CTA CCA TTC AAC TCC CGA GTA CAT AGA GTG TTT T	46,565,034 (chr 15)	Intron 29	0.36
D15S123	AGC TGA ACC CAA TGG ACT TTT CAT GCC ACC AAC AAA	45,851,813 (chr 15)	3'	0.81
D15S143	AAT TGA TTA GCC TAC CCA CTG TCC TAG ACT CCT ACA TGT TTG T	45,690,603 (chr 15)	3'	0.64

* With respect to *FBNI* gene.

mean age 57 ± 13 years, and mean disease duration 8 ± 7 years; patients' characteristics are given in Table 2. The control group contained 156 healthy subjects matched for age and sex with SSc patients. The Italian cohort regrouped 266 SSc patients and 153 healthy controls (Table 2).

The enlarged French cohort included 362 French SSc patients and 162 matched controls; the characteristics of this new enlarged cohort were similar to the initial cohort (Table 2).

Hardy-Weinberg equilibrium and linkage disequilibrium.

All the polymorphisms were in Hardy-Weinberg equilibrium for both the patient and control groups. Allelic association analysis revealed 2 sets of markers in close LD for the

first 6 markers tested. The first contained the 4 markers (D15S1028, intron C, MTS2, and MTS3) in the 5' region of the *FBNI* gene and the second contained the 2 markers (D15S123, D15S143) in the 3' region (Figure 1). As shown in Figure 1, in the first set of markers in LD, all the markers were in LD ones with the others. We found similar LD patterns in the initial French and Italian cohorts. However, allelic frequencies of the enlarged French and Italian controls were different, thus we were unable to pool these 2 cohorts into one.

Association between microsatellite alleles, intron C SNP, and SSc. We genotyped all subjects from the initial French cohort for the 5 microsatellites and the intron C SNP, obtain-

Table 2. Characteristics of the French and Italian Caucasian cohorts of patients with SSc. Data are number of patients (%).

Characteristic	Initial French SSc Cohort, n = 243	Additional SSc Patients, n = 163	Enlarged French SSc Cohort, n = 362	Italian SSc Cohort, n = 266
Age, yrs ± SD	57 ± 13	59 ± 12	57 ± 13	58 ± 12
Sex female	209 (86)	145 (89)	311 (86)	240 (90)
Disease duration, yrs ± SD	8 ± 7	10 ± 7	9 ± 8	10 ± 8
Diffuse cutaneous form	98 (40)	55 (34)	135 (37)	91 (34)
Pulmonary fibrosis	111 (45)	78 (48)	173 (48)	111 (42)
Forced vital capacity < 75% predicted value	37 (15)	28 (17)	54 (15)	55 (21)
DLC0/VA < 75%	87 (36)	65 (40)	135 (37)	140 (53)**
Pulmonary arterial hypertension	19 (8)	14 (9)	33 (9)	30 (11)
Renal crisis	3 (1)	1 (1)	4 (1)	4 (2)
Positive antinuclear antibodies	204 (84)	155 (95)	319 (88)	253 (95)
Positive antitopoisomerase I antibodies	61 (25)	49 (30)	94 (26)	64 (24)
Positive anticentromere antibodies	63 (26)	73 (45)*	121 (33)	151 (57)**

* p < 0.05 between new SSc patients and the initial cohort. ** p < 0.05 between the Italian cohort and the 2 French cohorts.

ing 2394 genotypes. No association was detected between alleles of these polymorphisms and the disease. The results for alleles with a frequency higher than 5% between SSc patients and controls are listed in Table 3. This analysis was carried out for various subgroups of patients having one disease characteristic, and no associations were identified. We

also looked at haplotypes taking into account all alleles, rare or not, constructed using the maximum likelihood procedure without highlighting any differences.

In the Italian cohort, allelic frequencies of the various markers were similar in the 266 SSc patients and the 153 matched controls (Table 3). Each subtype phenotype was

Table 3. Allelic frequencies of microsatellite markers and intron C SNP in the initial French and Italian cohorts. No significant differences for comparisons between French SSc patients and French controls, or between Italian SSc patients and Italian controls. Data are number of alleles (%).

Marker	Allele	French SSc, n = 243	French Controls, n = 156	Italian SSc, n = 266	Italian Controls, n = 153
D15S1028	171	34 (7)	17 (5.5)	35 (6.6)	14 (4.6)
	173	30 (6.2)	29 (9.2)	16 (3)	16 (5.2)
	175	153 (31.6)	102 (32.7)	150 (28.2)	104 (34)
	177	43 (8.9)	22 (7)	44 (8.3)	21 (6.9)
	179	55 (11.4)	39 (12.5)	92 (17.3)	36 (11.8)
	181	125 (25.8)	80 (25.6)	138 (25.9)	77 (25.2)
Intron C	T	326 (68.5)	214 (72.8)	377 (70.9)	225 (73.5)
	C	150 (31.5)	80 (27.2)	155 (29.1)	81 (26.5)
MTS2	137	35 (7.5)	25 (8)	46 (8.6)	30 (9.8)
	145	249 (53.7)	173 (55.8)	304 (57.1)	167 (54.6)
	156	27 (5.8)	18 (5.8)	21 (3.9)	14 (4.6)
MTS3	160	48 (10.3)	29 (9.4)	43 (8.1)	26 (8.5)
	146	77 (16)	39 (12.5)	41 (7.7)	28 (9.1)
	151	41 (8.5)	26 (8.3)	55 (10.3)	32 (10.5)
	156	362 (75.4)	247 (79.2)	436 (81.9)	245 (80)
D15S123	191	30 (6.2)	18 (5.8)	11 (2.1)	16 (5.2)
	193	169 (35)	106 (34.2)	218 (41)	107 (35)
	197	27 (5.6)	28 (9)	35 (6.6)	17 (5.6)
	199	70 (14.5)	43 (13.9)	96 (18)	44 (14.4)
	201	124 (25.7)	71 (22.9)	125 (23.5)	82 (26.8)
D15S143	190	154 (32.9)	98 (31.8)	196 (36.8)	101 (33)
	196	37 (7.9)	28 (9.1)	59 (11.1)	34 (11.1)
	198	216 (46.1)	158 (51.3)	221 (41.5)	134 (43.8)
	200	41 (8.8)	18 (5.8)	41 (7.7)	24 (7.8)

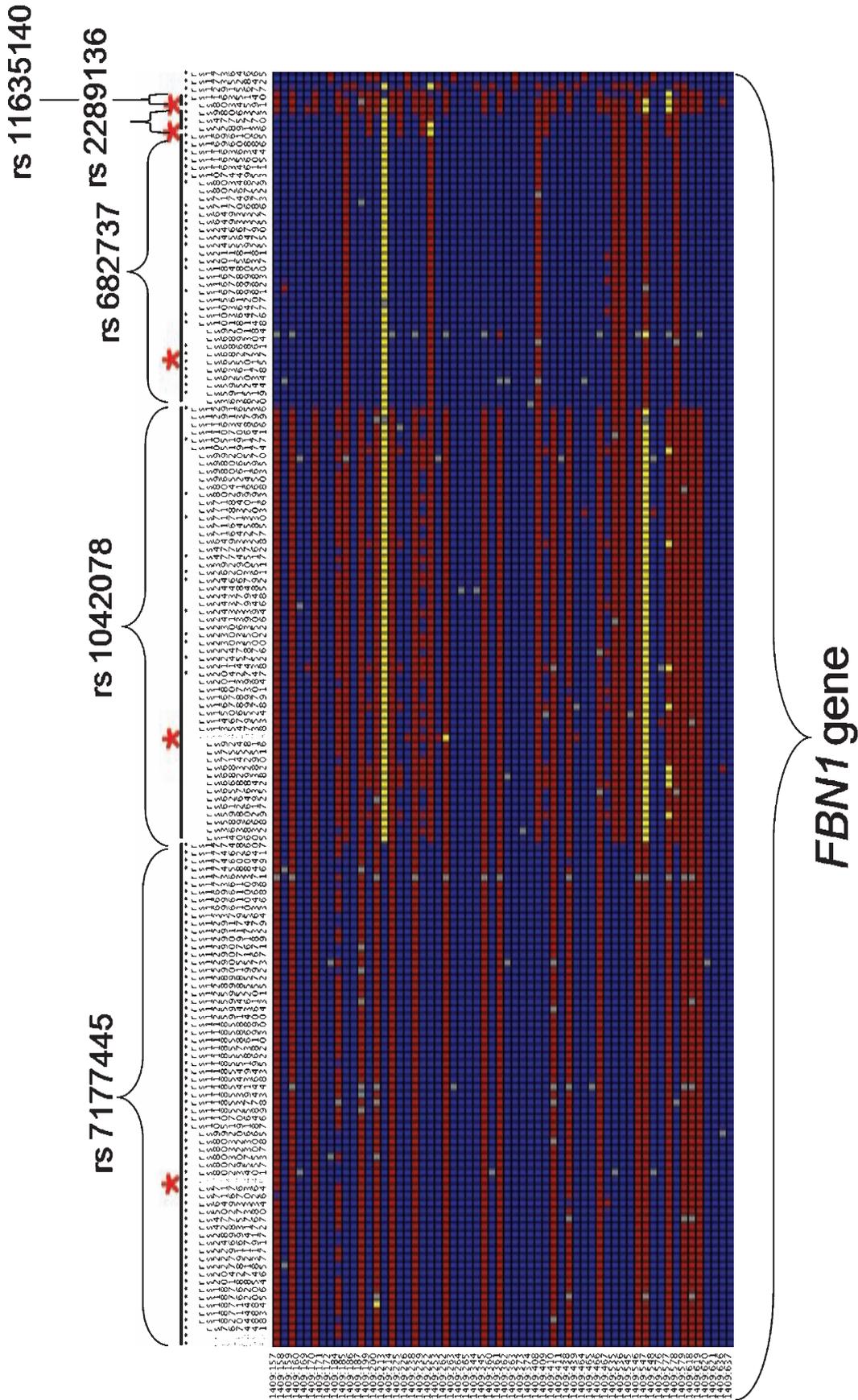


Figure 2. Genotype diagram at the *FBN1* locus. Each row corresponds to a DNA sample from one of 60 unrelated individuals of the phase II HapMap-CEU cohort, each column corresponds to a common polymorphic site. Sites are ordered by linkage disequilibrium, not by position, along the chromosome, with sites showing similar genotype patterns placed adjacent to one another. Selected SNP are indicated with red stars at the top. The graphic tool used was VG2 (<http://pga.gs.washington.edu/vg2.html>). *Location of the assessed tagSNP in each *FBN1* subregion in linkage disequilibrium.

considered for analysis; again there were no differences between the subpopulations and the controls. No haplotype was associated with SSc or any subgroup of patients.

Association between *FBNI* tagSNP and SSc. TagSNP analysis was performed in the larger French cohort to confirm the results of the first part of the study using various markers that cover the entire *FBNI* gene. No significant evidence of allelic association was observed for the SNP. Frequencies of minor alleles of each SNP were similar for SSc patients and controls: 12% versus 14%, respectively, for the C allele of SNP1 ($p = 0.25$), 16% versus 12% for the C allele of SNP2 ($p = 0.25$), 12% versus 12% for the T allele of SNP3 ($p = 0.88$), 26% versus 24% for the C allele of SNP4 ($p = 0.52$), and 31% versus 31% for the C allele of SNP5 ($p = 0.97$). The details of frequencies are given in Table 4. Allele frequency and haplotype reconstruction revealed no significant differences between the subgroups of patients tested.

DISCUSSION

There is increasing evidence that abnormalities in fibrillin-1 may play an important role in SSc pathogenesis¹³. Our study did not detect any association between certain alleles of polymorphic *FBNI* gene markers and the disease, focusing on a European Caucasian population. Previous genetic studies had focused only on Native American and Japanese patients. A common *FBNI*-containing haplotype suspected to be inherited from common ancestors was found in Choctaws⁷. An associated SNP in the 5' region of the gene was also identified from this population⁸. In a Japanese population, a negative association between SSc and another SNP of the 5'-UTR was found⁹. Thus we carried out case-control studies using a much larger sample compared to previous studies evaluating *FBNI* involvement⁷⁻⁹.

Two types of complementary polymorphic markers were used to replicate these results in our cohorts. First, we per-

formed microsatellite genotyping. These markers have been used previously in SSc studies⁷, and have also proved to be informative in familial studies of Marfan syndrome. Then we investigated 5 SNP to refine the map of the *FBNI* gene using HapMap data and haplotype reconstruction, avoiding markers with numerous alleles. There was no association between the polymorphic markers of our European Caucasian SSc patients and the disease, including its related complications. The 5 SNP would have been genotyped in the Italian cohort, but we did not investigate these markers here, taking into account the previous negative results — the absence of association with microsatellites in this Italian cohort and with the 5 tagSNP in the enlarged French cohort.

The absence of an association could mean 2 things: either the association does not exist or we were unable to detect it. For the first hypothesis, it must be emphasized that we used larger sample sets than previous studies, and 2 sets of complementary polymorphic markers; for the second, there are several possible explanations for why we may not have detected an existing association. The estimated sample size required for association studies in SSc was a minimum of 1200 patients, which is more than we included, and as many controls as needed to obtain 80% power to detect an association at $\alpha = 10^{-5}$, as described¹. Investigation of multiplex families, or at least case-parent trios, would allow a more robust approach in determining the genes involved; however, the late onset of the disease and its very low prevalence preclude this.

Heterogeneity may also affect the detection of an association. No Mendelian inheritance pattern has been attributed to SSc, although polygenic effects similar to those seen in other autoimmune disorders suggest a complex pattern of inheritance. Evidence supporting a genetic background for SSc is provided by addition of the small effects of each of the implicated loci and polymorphic alleles. This is one of the principal differences between the Caucasian population and the Native American Choctaw patients, who have a different and far more homogeneous genetic background, since this population experienced a bottleneck in the 19th century. The result is that in the Choctaw population, the homogeneous phenotype probably reflects the expression of fewer variants, which consequently have a more pronounced genetic effect that is easier to detect. Heterogeneity in Caucasians with SSc, characterized by the multiplication of clinical subphenotypes, dilutes the genetic effect of each individual variant. This may be why we were unable to detect an association between polymorphisms and the disease or any patient subgroup.

Finally, it is possible that the role of *FBNI* in SSc is not based on molecular mechanisms but perhaps on posttranscriptional or posttranslational mechanisms¹³. This kind of mechanism would be supported by polymorphisms other than those assessed here because of our negative results. Fibrillin-1 is a cysteine-rich glycoprotein, and recent studies

Table 4. Allelic frequencies of single-nucleotide polymorphisms in the enlarged French cohort. No significant difference was found for comparisons between the enlarged group of French patients with SSc and French controls. Data are number of alleles (%).

Polymorphism	Allele	Enlarged French SSc Cohort, n = 362	Enlarged French Control Group, n = 162	p*
rs 7177445	A	637 (88)	278 (86)	0.25
	C	87 (12)	46 (14)	
rs 2289136	T	608 (84)	285 (88)	0.25
	C	116 (16)	39 (12)	
rs 682737	C	637 (88)	285 (88)	0.88
	T	87 (12)	39 (12)	
rs 1042078	T	536 (74)	246 (76)	0.52
	C	188 (26)	78 (24)	
rs 11635140	T	500 (69)	224 (69)	0.97
	C	224 (31)	80 (31)	

* Comparison between enlarged French SSc patient cohort and controls.

had revealed that fibrillin-1 synthesized by human SSc fibroblasts may be unstable¹⁰. The quantitative defect of FBN-1 seems to be a consequence of qualitative abnormalities, with an increased instability of fibrillin-1-containing microfibrils, rather than of quantitative abnormalities associated with decreased expression of the gene. Finally, published data are consistent with fibrillin-mediated TGF- β signaling abnormalities¹⁴. In Marfan syndrome, fibrillin-1 defects are thought to impair sequestration of the latent form of TGF- β in the extracellular matrix and to increase TGF- β activity⁶; moreover, *TGBR2* was found to be the second major gene involved in the disease¹⁵. Interactions between defective fibrillin-1 and the TGF- β pathway may contribute to fibrosis and vasculopathy in SSc.

Our findings are in contrast to genetic results obtained from the Choctaw and Japanese populations of patients with SSc. We suggest that these discrepancies can be explained by heterogeneity associated with this multigenic disease. The structural and functional defects observed in Choctaw patients should be investigated using fibroblasts from Caucasian patients. Finally, the relationship between fibrillin-1 and TGF- β signaling should be investigated further.

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