Association study of 3 rheumatoid arthritis risk loci in systemic sclerosis in a European Caucasian population

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ABSTRACT

Introduction. *Accumulating evidences show that shared autoimmunity is critical for the pathogenesis of many inflammatory rheumatic conditions. Specific phenotype could arise from specific genes, and/or combination of genetic factors and environment. Systemic sclerosis (SSc) belongs to connective tissue disorders and recent data have highlighted strong associations with some autoimmunity genes shared with other autoimmune diseases.*

Objective. *To determine whether novel risk loci associated with rheumatoid arthritis (RA) may confer susceptibility to SSc. Single nucleotide polymorphism from CCL21, CD244 and CDK6 were tested for association.*

Patients and methods. *SNPs harbouring association with RA, CCL21-rs2812378, CDK6-rs42041 and CD244-rs6682654 were genotyped in a cohort of 1031 SSc patients and 1014 controls. All individuals were of European Caucasian origin.*

Results. *The three polymorphisms were in Hardy-Weinberg equilibrium in the control population and allelic frequencies were similar to those expected in European populations. Allelic and genotypic frequencies for these three polymorphisms were found to be similar in SSc patients and controls. Moreover, sub-phenotype analyses in particular for subgroups having diffuse subcutaneous subtype, specific auto-antibodies or fibrosing alveolitis did not detect any difference between SSc patients and controls.*

Conclusion. *These results obtained through a large cohort of European Caucasian SSc patients do not support the implication of CCL21, CD244 and CDK6 genes in the pathogenesis of SSc although these genes were recently identified as RA susceptibility genes.*

Introduction

Systemic sclerosis (SSc) is a chronic autoimmune disease with a complex pathogenesis that is driven by combination of genetic risk factors and environmental events (1).

Circulating autoantibodies, infiltration of mononuclear cells in affected organs and altered immune mediators are well-known features of SSc. Scleroderma skin is also characterised by an early infiltration of T cells, mainly perivascular, before fibrotic changes (2). The recruitment of T cells in targeted tissues involves chemokines and NK cells may also be important for contributing to early host defense and could represent a link with adaptative immunity (3). Accumulating data have demonstrated shared autoimmunity pathways and susceptibility factors between various autoimmune diseases (4), indicating that they provide a lowering of the background threshold for the development of pathogenesis. Shared autoimmunity suggests the involvement of a particular cluster of genetic and environmental susceptibility factors. Most of susceptibility genetic factors are frequently replicated in different diseases such as diabetes mellitus, multiple sclerosis, juvenile idiopathic arthritis, celiac disease. Furthermore, several autoimmune diseases are often associated with typical clustering in organ-specific disease or multisystem (5).

The well-known main shared genetic factors which contribute the most to SSc susceptibility are Major histocompatibility complex (*MHC*), *STAT4* and *IRF5* (6). These genes and pathways are also known for contributing to rheumatoid arthritis (RA) susceptibility. In this latter disease, some new risk loci have been recently identified. Indeed, rs 2812378 variant located in *CCL21* that encodes for a chemokine which plays

a role by stimulating the chemotaxis in particular of activated T cells and by inhibiting haematopoiesis, has been found to contribute to RA genetic susceptibility in a Genome-wide association study (OR of 1.1) (7). It was also associated with disease severity (8) and this has been replicated in independent cohorts (9, 10). *CD 244* is a membrane receptor, member of the signalling lymphocyte activation molecule (SLAM) expressed on natural killer cells and that modulate the non-major histocompatibility complex (MHC) restricted killing. The rs6682654 variant has been found to contribute to RA genetic susceptibility in a gene candidate study (Japanese cohort) with OR at 1.34 (11). *CDK6* is a cyclin-dependant kinase that controls progression through the cell cycle and initiation of differentiation in many cells. The rs42041 variant has been found to contribute to RA genetic susceptibility in a gene candidate study with an OR at 1.15 (10).

Taking into account i) the autoimmune background of SSc ii) the contribution of shared autoimmunity in this severe condition iii) the very recent report of new autoimmune susceptibility risk factors for RA, we investigated whether *CCL21*, *CD244* and *CDK6* may confer susceptibility to SSc.

Patients and methods

We performed a large case-control association study in a cohort consisting of 1031 SSc patients and 1014 healthy unrelated controls, all from French European Caucasian origin. For all SSc patients, we determined LeRoy's cutaneous subtype (12) and carried out a phenotypic assessment (13), as recommended.

The study was approved by our institutional review board, and written informed consent was obtained from all subjects. For testing autoimmune subgroup in SSc, all patients were tested for antinuclear antibodies by indirect immunofluorescence in their local centers. We systematically checked for antibodies specific to SSc: Anti-centromere antibodies (ACA) based on their distinctive IIF pattern and Anti-topoisomerase I antibodies by counter immunoelectrophoresis. Demographic data and disease characteristics of SSc patients are detailed in Table I.

Table II. Characteristics of the systemic sclerosis patients.

Patients n (%)	SSc cohort (n=1031)
Age (years±SD)	46.2±14.8
Sex (female %)	85.7
Disease duration (years±SD)	10.6±8.1
Diffuse cutaneous sub-type n (%)	321/971 (33)
Limited cutaneous sub-type n (%)	650/971 (67)
Anti-nuclear Abs (≥1/160) n (%)	739/928 (80)
Positive anti-topoisomerase I Abs n (%)	253/928 (27)
Positive anti-centromere I Abs n (%)	377/928 (41)
Fibrosing alveolitis on CT-scan	359/920 (39)
Pulmonary arterial hypertension	68/965 (7)
Digital ulcers (past or present)	333/918 (36)

Abs: antibodies; n: number; SD: standard deviation.
SSc associated fibrosing alveolitis was defined as presence of typical features on chest high resolution computerised tomography (HRCT), pulmonary hypertension (PAH) was defined as precapillary PAH on catheterisation and digital ulcers included both current and past history of ulcerations.

Genotyping

We selected the following three SNPs for which convincing association was reported with RA: rs2812378, rs42041 and rs6682654. The 3 SNPs were genotyped using a competitive allele specific PCR system (Kaspar genotyping, Kbioscience, Hoddeston, UK) as previously reported (14). The average genotype completeness for these SNPs polymorphisms was 99% for the SSc and the control samples. The accuracy was >99%, according to duplicate genotyping of 10% of all samples using the Taqman SNP genotyping assay-allelic discrimination method (Applied Biosystem, Foster City, CA).

Statistical analyses

The statistical analyses were performed using the R computer package software (version 2.6.0). The level of significance for all the tests corresponds to a type-I error-rate α=5%. Tests for conformity with Hardy-Weinberg equilibrium were performed using a standard χ^2 test (1 degree of freedom d.f.). Individual association analyses of the 3 SNPs with SSc were performed by comparing cases and controls with a Fisher's exact test on genotype distribution. The same procedure was applied in subgroups stratified according to SSc phenotypes.

Power calculation

Power was assessed by a standard non-central chi-square approximation, as previously described (14). Taking into account the expected frequency of the rare allele of rs2812378, the set has respectively a power of 99.3% and 80.8% for detecting an association between SSc and this *CCL21* variant, with an OR of 1.5 and 1.3, at the 5% significance level. For *CDK6* rs42041, the set has a power of 98.6% and 75.7%, respectively, with an OR of 1.5 and 1.3. For *CD244* rs6682654, the set has a power of 99.5% and 83.2%, respectively, with an OR of 1.5 and 1.3.

Results

All the SNPs were at Hardy-Weinberg equilibrium in the control population. Allelic frequencies were found in good agreement with those already reported in the general European population.

The *CCL21* rs2812378 C allele was found on 31% of chromosomes from SSc cases compared to 33% of chromosomes from the controls (p=0.19) (Table II). The *CD244* rs6682654 G minor allele was found on 46% of chromosomes from SSc cases and did not deviate with the frequency observed in the controls (44%; p=0.11) (Table II).

Regarding the third locus, the *CDK6* rs42041 G allele was found on 26% of chromosomes fom SSc cases compared to 26% of chromosomes from the controls (p=0.71) (Table II).

No significant evidence of allelic or genotypic association was therefore detected for the *CCL21*, *CD244* and *CDK6* SNPs (Table II). Furthermore, regard-

Table II. Frequencies of CCL21, CD244 and CDK6 alleles and genotypes in SSc, in the diffuse and limited cutaneous subsets, in SSc patients with anti-centromere or anti-topoisomerase antibodies and controls.

CCL21 rs2812378	n. (%)						
	CC	CT	TT	*p*-value	C	T	*p*-value
Controls	113 (11)	440 (44)	455 (45)	–	666 (33)	1350 (67)	–
SSc	96 (9)	444 (44)	482 (47)	0.15	636 (31)	1408 (69)	0.19
dcSSc	21 (7)	150 (48)	140 (45)	0.05	192 (31)	430 (69)	0.31
lcSSc	58 (9)	268 (42)	317 (49)	0.08	384 (30)	902 (70)	0.06
ACA	33 (9)	162 (43)	178 (48)	0.18	228 (31)	518 (69)	0.22
TOPO	19 (8)	108 (43)	124 (49)	0.07	146 (29)	356 (71)	0.09
CD244 rs6682654	n. (%)						
	GG	GA	AA	*p*-value	G	A	*p*-value
Controls	185 (19)	487 (49)	312 (32)	–	857 (44)	1111 (56)	–
SSc	216 (21)	499 (50)	295 (29)	0.10	931 (46)	1089 (54)	0.11
dcSSc	69 (22)	154 (49)	90 (29)	0.16	292 (47)	334 (53)	0.17
lcSSc	132 (21)	316 (49)	191 (30)	0.30	580 (45)	698 (55)	0.30
ACA	84 (23)	172 (46)	116 (31)	0.24	340 (46)	404 (54)	0.31
TOPO	51 (21)	118 (48)	78 (31)	0.63	220 (45)	274 (55)	0.69
CDK6 rs42041	n. (%)						
	GG	GC	CC	*p*-value	G	C	*p*-value
Controls	66 (6)	386 (39)	546 (55)	–	518 (26)	1478 (74)	–
SSc	71 (7)	394 (39)	550 (54)	0.66	538 (26)	1494 (74)	0.71
dcSSc	29 (9)	107 (34)	181 (57)	0.24	165 (26)	469 (74)	0.97
lcSSc	40 (6)	259 (41)	340 (53)	0.90	339 (27)	939 (73)	0.72
ACA	22 (6)	148 (40)	199 (54)	0.73	192 (26)	546 (74)	0.97
TOPO	21 (8)	94 (38)	133 (54)	0.32	136 (27)	360 (73)	0.51

SSc: systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis; lcSSc: limited cutaneous systemic sclerosis; ACA: anti-centromere antibodies; TOPO1: anti-topoisomerase antibodies.

ing SSc sub-phenotypes, intra-cohort comparisons also failed to detect any association. The results, detailed in Table II, include those in one sub-phenotype with the more pronounced fibrotic propensity that is the subset of patients with the diffuse cutaneous forms.

Discussion

Although rare, SSc presents a major medical challenge, being recognised as the most severe connective tissue disorder in terms of its prognosis (15). Molecular biology has provided unparalleled insight into the susceptibility genes conferring a predisposition to this disease and has improved our understanding of its complex immune pathogenesis.

Shared autoimmunity pathways between SSc and RA are well illustrated by common genetic susceptibility factors. Some of them are particularly involved in innate immunity such as *IRF5* and *STAT4* and also in adaptative immunity with MHC and *PTPN22*.

Although there are many shared susceptibility factors, some differences could differentiate the pathogenesis between these diseases. In RA, very recent data have highlighted new pathways in particular in initiation of inflammatory response, with a suggested key link between innate and adaptative immunity. NK cells could interact with T cells by a receptor CD244. A recruitment of local T cell involves chemokines and pathways of cell proliferation.

This led us to test for association these new loci in SSc. Our results obtained in a large cohort show that the included polymorphisms of *CCL21*, *CD244* and *CDK6* do not contribute to susceptibility of SSc or its subphenotypes, in particular subgroups having diffuse cutaneous subtype and fibrosing alveolitis. Methodological limitations of genetic studies must always be considered. Appropriate sample sizes for case and control cohorts are critical to provide sufficient statistical power. In this study, the large sample size of the cohort allowed us providing a strong power higher than 98% for detecting association with OR at 1.5; however our study cannot rule out the possibility that even larger numbers of cases and controls would be necessary to detect association with weaker OR. Moreover, the genetic background of the studied population should be as homogenous as possible, thereby limiting bias by population stratification. To avoid this bias ethnicity was taken into account and we have focused on European Caucasian individuals. Finally, allelic and genotypic frequencies in our controls were found in agreement with previously reported frequencies in European Caucasian populations. Our results rise that although shared auto-immunity seems to strongly contribute to the susceptibility of many inflammatory rheumatic conditions and auto-immune diseases, some more specific genetic or other factors probably act to generate the respective phenotypes. Neverthe-

less, more SNP markers at these loci should be investigated before definitely ruling out association with SSc.

In conclusion, the genotyping of three RA risk loci (*CCL21*, *CD244* and *CDK6*) in a large cohort of European Caucasian SSc patients did not allow the detection of any allelic or genotypic association with the disease or its main subsets.

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