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Association study of B-cell marker gene polymorphisms in European Caucasian patients with systemic sclerosis

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ABSTRACT

Background. BANK1 *and* BLK *B-cell genetic markers have been reproducibly and convincingly found to contribute to susceptibility to systemic sclerosis (SSc).*

Objectives. *To determine whether other B-cell genetic markers including* CD19, CD20, CD22 *and* CD24 *polymorphisms affect susceptibility to SSc in the European Caucasian population.*

Methods. *A case-control study was performed in 900 patients with SSc and 1034 healthy controls. Among the whole SSc population, 304 (34%) had the diffuse cutaneous subtype, 551 (61%) had the limited cutaneous subtype, 732 (81%) were positive for antinuclear antibodies , 331 (37%) were positive for anticentromere antibodies and 228 (25%) for the topo-isomerase I. Genotyping has been performed for CD19 rs35979293, CD19 rs2904880, CD20 rs7126354, CD20 rs3802954, CD20 rs105146, CD20 rs4939364, CD22 rs10406069, CD22 rs10413500, CD22 rs10419538, CD22 rs34826052 and CD24 ins-del polymorphisms.*

Results. *Genotype frequencies were at the Hardy-Weinberg equilibrium in the control population for all the SNPs investigated and observed frequencies were very similar to those expected in the European population. Allelic and genotypic frequencies for all these tested SNPs were found to be similar in SSc patients and controls. Moreover, subphenotype analyses in particular for subgroups having the diffuse cutaneous subset or topo-isomerase I positive antibodies, which are the most associated with* BANK1 *variants, did not detect any difference between SSc patients and controls.*

Conclusion. *These results obtained through a large cohort of European caucasian patients with SSc do not support the contribution of* CD19, CD20, CD22, CD24 *variants to the genetic susceptibility of SSc.*

Introduction

Systemic sclerosis (SSc) is a severe multiorgan disease with complex pathogenesis. There is now evidence to support the hypothesis that SSc is an autoimmune process modulated by both environmental and genetic factors (1-3). The exact molecular basis for SSc is still unknown. However, several lines of evidences have suggested a key role for B-cells. Indeed, the detection of specific circulating auto-antibodies including anti-topoisomerase I, anti-centromere and anti-RNA polymerase antibodies, is a common identifying feature which usually precedes disease onset. The B-cell stimulator, BAFF, has been shown to be increased in the serum mainly in patients having dcSSc and to correlate with the skin score (4). In the *tight skin* mouse, a genetic model for human SSc, alterations in CD19-dependent signalling pathways may contribute to both skin fibrosis and systemic auto-immunity (5). Rituximab seems to be beneficial in this animal model and very recent preliminary data suggest that rituximab may favourably affect the outcomes of human disease (6). Lastly, these data have been strengthened by large genetic studies showing *BANK1* gene, encoding for a B-cell specific scaffold protein, and more recently, *BLK* locus, encoding for B-lymphoid tyrosine kinase, as genetic susceptibility factors respectively in diffuse cutaneaous SSc (dcSSc) for *BANK1* (7), and regardless of the clinical subset for *BLK* (8). Among B-cell regulators, CD19, CD20, CD22 ad CD24 seem to play a role in B-cell activation: CD19, a B-cell specific signal transduction molecule, has been shown to be over-expressed in peripheral blood cells from SSc patients (5); CD20, a B-cell-surface specific protein, is involved in B-cell cycle initiation and differentiation (6); CD22 mediates B-cell B-cell interactions and is involved in lymphoid tissues B-cells localisation; finally, CD24 a sialoglycoprotein expressed on mature granulocytes and many B-cells, modulates B-cell activation responses (13). Preliminary studies have also suggested that *CD19* and *CD22* variants may contribute to the limited cutaneous subset of SSc (9, 12). Of note, variants of B-cell markers have been identified as susceptibility factors for some other autoimmune disorders including *CD19* in rheumatoid arthritis (RA) and lupus (SLE) and *CD24* in SLE, multiple sclerosis (MS) and RA

(10-14). Deciphering B-cell contribution in SSc is critical with regards to the recent development of anti-CD20 or anti-CD22 drugs in several autoimmune disorders. Therefore, the aim of this study was to test for association with SSc polymorphisms encoding for B-cell markers of the *CD19, CD20, CD22* and *CD24* genes.

Patients and methods

Patients

We performed a case-control association study in 900 patients with SSc and 1034 healthy controls from European Caucasian origin. Local institutional review boards approved the study, and written informed consent was obtained. All SSc patients were classified by cutaneous subtype according to Leroy *et al*. (15). We carried out a phenotypic assessment, as recommended. The control groups consisted of healthy unrelated individuals matched to the SSc cases for ethnicity (all subjects were of European Caucasian ancestry) and also for sex and age (85.2% women, mean age 52.2±8.4 years). All patients with SSc were tested for antinuclear antibodies by indirect immunofluorescence (IIF), with HEp-2 cells as the antigen substrate (Antibodies Inc., Davis, CA). We systematically checked for antibodies specific to SSc. Anti-centromere antibodies (ACAs) were identified on the basis of their distinctive IIF pattern. Anti-topoisomerase I antibodies were determined by counter immunoelectrophoresis. With regards to the assessments of vascular phenotype, precapillary pulmonary arterial hypertension (PAH) was diagnosed by catheterisation. Pulmonary fibrosing alveolitis was defined as the presence of typical features on high-resolution computerised tomography (HRCT) of the chest, this procedure being carried out in all SSc patients.

Genotyping

We selected the following SNPs for which the most convincing associations with autoimmune diseases have been reported. All the SNPs tested in this study were TagSNPs in Caucasians: *CD19* rs35979293, *CD19* rs2904880, *CD20* rs7126354, *CD20* rs3802954, *CD20* rs105146, *CD20* rs4939364, *CD22* rs10406069, *CD22* rs10413500, *CD22* rs10419538, *CD22* rs34826052 and *CD24* ins-del polymorphism (9-14). Genotyping was performed using a competitive, allele-specific polymerase chain reaction system (Kaspar Genotyping, KBiosciences, Hoddesdon, UK). The average genotype completeness for these polymorphisms was 98% for the SSc patients and control samples. The accuracy was >99% according to duplicate genotyping of 10% of samples with the same technique.

Statistical Analyses

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) assessing the differences between observed genotype distributions and expected genotype distributions based on control population allele frequencies. Individual association analyses of the 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 (10 subsets). Corrections were applied for multiple testing using Bonferroni's correction: *p* values were multiplied by 11 for SNP association with the disease and by 10 when subphenotypes were tested. *P*-values after this adjustment for multiple testing are indicated in the tables as p_{adj}. The corresponding ORs were assessed by standard logistic regression analysis, with the most frequent genotype taken as the reference. Power calculation was assessed by a standard non-central chi-square approximation, as an example, taking into account the expected frequency of *CD19* rs2904880 (26%), the set has a power >90% for detecting association with SSc, for an OR of 1.5, at the 5% significance level.

Table I. Characteristics of the SSc population studied.

Patients	SSc cohort, n=900
Women, n (%)	751 (83%)
Mean age, (years ± SD)	57 ± 13
Mean disease duration, (years ± SD)	12 ± 9
Diffuse cutaneous subtype (dcSSc), n (%)	304 (34%)
Limited cutaneous subtype (lcSSc), n (%)	551 (61%)
Antinuclear antibodies (ANAs), n (%)	732 (81%)
Anti centromere antibodies (ACAs), n (%)	331 (37%)
Anti topo-isomerase I antibodies, n (%)	228 (25%)

Results

The demographic data and disease characteristics of patients with SSc are detailed in Table I. Genotype frequencies were at the Hardy-Weinberg equilibrium in the control population for all of the SNPs investigated. Allelic frequencies were found in good agreement with those already reported in the European population. The minor allele frequencies of all tested SNPs observed in the SSc patients did not differ from the frequency found in controls after correction for multiple testing (Table II). Furthermore, regarding SSc subphenotypes, 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: patients having the diffuse cutaneous form. We did not detect any association regarding the other subgroups according to antibodies status (ANAs, ACAs, Anti-topoisomerase I antibodies), the presence of fibrosing alveolitis, or the presence PAH (data not shown, available upon request from the author for correspondence).

Discussion

The aim of this study was to investigate B-cell markers as genetic susceptibility factors to SSc. We also investigated 2 *CD19* (rs35979293 and rs2904880), 4 *CD20* (rs7126354,

Table II. Genotype and allelic frequencies of the 11 B cell marker polymorphisms depending on cutaneous status.

	11 n. (%)	12 n. (%)	22 n. (%)	MAF	*p*-value		*p*-adj*	
					1	11	1	11
CD19rs35979293 T=1 G=2								
SSc	113 (13.3)	407 (47.8)	332 (39.0)	37.1	0.67	0.46	–	–
dcSSc	47 (16.2)	130 (44.7)	114 (39.2)	38.5	0.77	0.67	–	–
lcSSc	62 (12.0)	260 (50.1)	197 (38.0)	37.0	0.66	0.29	–	–
Controls	144 (14.9)	445 (45.9)	380 (39.2)	37.8				
CD19rs2904880 C=1 G=2								
SSc	52 (6.0)	344 (39.5)	476 (54.6)	25.7	0.30	0.14	–	–
dcSSc	13 (4.4)	110 (36.9)	175 (58.7)	22.8	0.03	0.03	0.3	0.3
lcSSc	36 (6.8)	216 (40.8)	278 (52.5)	27.2	0.99	0.60	–	–
Controls	77 (7.7)	388 (38.9)	532 (53.4)	27.2				
CD20rs7126354 C=1 T=2								
SSc	112 (12.9)	417 (47.9)	340 (39.1)	36.9	0.96	0.84	–	–
dcSSc	31 (10.6)	132 (45.1)	130 (44.4)	33.1	0.09	0.11	–	–
lcSSc	76 (14.2)	263 (49.1)	197 (36.8)	38.7	0.34	0.44	–	–
Controls	135 (13.4)	476 (47.2)	398 (39.4)	37.0				
CD20rs3802954 G=1 A=2								
SSc	6 (0.7)	90 (10.5)	762 (88.8)	5.9	0.047	0.42	0.47	–
dcSSc	2 (0.7)	26 (9.0)	262 (90.3)	5.2	0.04	0.57	0.4	–
lcSSc	3 (0.6)	59 (11.2)	463 (88.2)	6.2	0.15	0.7	–	–
Controls	4 (0.4)	144 (14.4)	853 (85.2)	7.6				
CD20rs105146 C=1 T=2								
SSc	104 (11.9)	376 (43.3)	388 (44.7)	33.6	0.78	0.93	–	–
dcSSc	42 (14.3)	129 (14.0)	122 (41.6)	36.4	0.16	0.25	–	–
lcSSc	58 (10.9)	233 (43.8)	241 (45.3)	32.8	0.81	0.50	–	–
Controls	126 (12.5)	417 (41.4)	464 (46.1)	33.2				
CD20rs4939364 A=1 G=2								
SSc	97 (11.0)	360 (40.9)	423 (48.1)	31.5	0.83	0.84	–	–
dcSSc	30 (10.1)	112 (37.7)	155 (52.2)	29.0	0.31	0.65	–	–
lcSSc	62 (11.5)	224 (41.6)	253 (46.9)	32.3	0.52	0.45	–	–
Controls	104 (10.3)	422 (41.7)	485 (47.9)	31.2				
CD22rs10406069 A=1 G=2								
SSc	565 (70.4)	208 (25.9)	30 (3.7)	16.7	0.11	0.78	–	–
dcSSc	188 (63.3)	99 (33.3)	10 (3.37)	20.0	0.47	0.85	–	–
lcSSc	352 (65.8)	165 (30.8)	18 (3.4)	18.8	0.96	0.71	–	–
Controls	667 (66.4)	300 (29.9)	38 (3.8)	18.7				
CD22rs10413500 1=G 2= C								
SSc	670 (76.2)	195 (22.2)	14 (1.6)	12.7	0.095	0.15	–	–
dcSSc	220 (74.1)	73 (24.6)	4 (1.4)	13.6	0.57	0.25	–	–
lcSSc	415 (77)	114 (21.2)	10 (1.9)	12.4	0.10	0.37	–	–
Controls	741 (73.4)	244 (24.2)	25 (2.5)	13.7				
CD22rs10419538 T=1 G=2								
SSc	636 (76.0)	186 (22.2)	15 (1.8)	12.9	0.46	0.30	–	–
dcSSc	224 (78.1)	59 (20.6)	4 (1.4)	11.7	0.20	0.24	–	–
lcSSc	384 (75.3)	117 (22.9)	9 (1.8)	13.2	0.70	0.37	–	–
Controls	751 (75.0)	225 (22.5)	25 (2.5)	13.7				
CD22rs34826052 G=1 T=2								
SSc	829 (93.9	53 (6.0)	1 (0.1)	3.1	0.92	0.29	–	–
dcSSc	282 (94.6)	15 (5.0)	1 (0.3)	2.8	0.7	–	–	–
lcSSc	504 (93.2)	37 (6.8)	0 (0.0)	3.4	0.52	0.08	–	–
Controls	946 (93.7)	64 (6.3)	0 (0.0)	3.2				
CD24 ins-del ins=1 del=2								
SSc	729 (84.3)	131 (15.1)	5 (0.6)	8.1	0.96	0.39	–	–
dcSSc	252 (85.7)	40 (13.6)	2 (0.7)	7.5	0.58	0.67	–	–
lcSSc Controls	441 (83.2)	86 (16.2)	3 (0.6)	8.7	0.65	0.46	–	–
Controls	815 (84.5)	140 (14.5)	9 (0.9)	8.2				

dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc. P adj*: P adjusted after Bonferroni correction. *p*-values are multiplicated by 11 in global SSc study (11 SNPs) and by 10 in different subgroups (10 subgroups tested).

rs3802954, rs105146, rs4939364), 4 *CD22* (rs10406069, rs10413500, rs10419538, rs34826052) SNPs and one *CD24* ins-del polymorphism. This is the first study investigating *CD19* and *CD22* polymorphisms in a SSc European population and also overall in SSc *CD20* and *CD24* polymorphisms. Using a very large population, no association was detected between SSc and the *CD19, CD20, CD22, CD24* variants investigated. Methodological limitations of genetic studies must always be considered. Many studies appear indeed statistically underpowered for the field of complex genetic diseases and are often shown to produce false-positive results. Appropriate sample sizes for case and control cohorts are required to provide sufficient statistical power. The large sample size of our cohort provided a strong power of detection, higher than 90% for ruling out type II error. Moreover, the genetic background of the study population should be as homogeneous as possible, thereby limiting bias by population stratification. To avoid these biases, ethnicity was taken into account and we have focused on individuals of European Caucasian origin. Finally, allelic and genotypic frequencies in our controls were found in agreement with the frequencies previously reported in European populations. Our results were obtained with European Caucasian populations and further replication studies are required to validate their extrapolation to other populations.

The association signal for *CD22* polymorphism that was previously suggested in the Japanese population was obtained through a small sample (126 SSc compared with 93 healthy controls) and without correction for multiple testing. (12). Our results therefore suggest that these non-replicated findings may be a type I error or that the previously detected association could be restricted to the Japanese population.

Regarding *CD19*, no association signal was observed in our Caucasian sample but the investigated SNPs variants were different to those previously tested in the Japanese population (9).

Therefore, an association in this latter population cannot be ruled out but independent replication is still warranted before drawing conclusion.

In conclusion, although B cells seem to exert an etiopathogenetic role in disease manifestations (4-8) our results obtained through a large cohort of European SSc patients do not support the contribution of *CD19, CD20, CD22, CD24* variants to the SSc genetic susceptibility, at least in the European Caucasian population.

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