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SHORT REPORT

Genetic Susceptibility to Systemic Sclerosis in the Greek-Cypriot Population: A Pilot Study

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Background: Systemic Sclerosis (SSc), also known as scleroderma, is an autoimmune rheumatic disease, which is clinically subdivided into two major subgroups; limited (lcSSc) and diffuse cutaneous scleroderma (dcSSc). Even though the SSc etiologies remains unclear, some HLA and non-HLA genetic variants have been associated with the disease.
Aim: This study was designed to evaluate the associations between several HLA-related genetic variants and SSc in the Greek-Cypriot population.
Methods: Forty-one SSc patients and 164 controls were genotyped at 18 selected single nucleotide polymorphisms (SNPs) using restriction fragment length polymorphism analyses, Sanger sequencing, and a multiplex SNaPshot minisequencing assay. Logistic regression analysis under the log-additive model was used to evaluate all possible associations between these SNPs and SSc; nominal statistical significance was assumed at $p < 0.05$.
Results: Associations of SSc with SNPs rs3117230, rs3128930, and rs3128965 within the *HLA-DPB1* and *HLA-DPB2* regions were observed in the Greek-Cypriot population at the level of $p < 0.05$. However, none of these associations survived a Bonferroni correction. The direction of the effect is consistent with the direction reported in previous studies. In addition, allele frequencies of the majority of the selected SNPs in the Greek-Cypriot population are similar to those reported in the European population.
Conclusion: This study initiates the genetic investigation of SSc in the Greek-Cypriot population, a relatively small newly investigated population. Further investigation with a larger sample size and/or additional SSc susceptibility loci may confirm the association of some of these variants with SSc in the Greek-Cypriot population that could potentially be used for predictive testing.

Keywords: systemic sclerosis, susceptibility loci, autoimmunity, population study

Introduction

Systemic sclerosis (SSc) is a rare autoimmune disease characterized by vasculopathies, inflammation, and fibrosis (Bossini-Castillo *et al.*, 2011; Allanore *et al.*, 2015). SSc is divided into two subgroups; limited (lcSSc) and diffused cutaneous SSc (dcSSc), based on the clinical manifestations. It occurs more frequently in women and onset is between the second and fifth decade of life (Alba *et al.*, 2014). Although the etiopathogenesis of SSc remains unclear, it is a multifactorial disease caused by a combination of genetic, epigenetic, and environmental factors (Broen *et al.*, 2014).

Many studies focused on the investigation of genetic factors and mechanisms that may be implicated in the triggering of the disease. Multiple genetic loci from the HLA as well as non-HLA regions have been associated with predisposition to SSc (Chairta *et al.*, 2017). Genome-wide association studies (GWAS) and HLA studies have shown that genetic variants

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in the HLA-Class II region are more frequently associated with the development of SSc, as compared with variants in the HLA-Class I and III regions (Radstake *et al.*, 2010; Allanore *et al.*, 2011; Chairta *et al.*, 2017). For example, the *HLA-DRB1*1104* allele, which is involved in HLA-Class II region was markedly associated with SSc in the Greek population (Vlachoyiannopoulos *et al.*, 2000).

In addition, single nucleotide polymorphisms (SNPs) in genes with different molecular functions like transient receptor potential melastatin channel genes (Oztuzcu *et al.*, 2015), Rho/Rho-kinase (Pehlivan *et al.*, 2016), Th17 pathway genes (Mellal *et al.*, 2018), and the Vitamin D receptor (Li *et al.*, 2019) were reported to be significantly associated with SSc susceptibility in the neighboring Turkish, Algerian, and Han Chinese populations, respectively.

SSc is genetically heterogeneous among populations and its prevalence in Europe ranges from 31 to 277 cases per million individuals (Silman *et al.*, 1988; Allcock *et al.*, 2004; Le Guern *et al.*, 2004; Alamanos *et al.*, 2005; Arias-Nunez *et al.*, 2008; Lo Monaco *et al.*, 2011). The prevalence of SSc in the Greek-Cypriot population has not been estimated and SSc susceptibility loci have not been evaluated.

Through this study, we aimed at the investigation of the Greek-Cypriot population for some already reported genetic variants that have been associated with SSc in other populations. The rationale of this study was to assess whether Greek-Cypriot patients with SSc have similar genetic susceptibility with patients from other populations, and evaluate whether some variants could be used in the future for prediction of the disease. In addition, this study initiated the collection of biomaterials and data for biobanking, as well as the prospective epidemiological investigation of SSc in our population.

Materials and Methods

Study participants

Forty-one patients with SSc were recruited for this study between February 2017 and January 2019, through their annual follow-up appointment at the Rheumatology Department of the Nicosia General Hospital. All patients fulfilled the 2013 American College of Rheumatology and the European League Against Rheumatism classification criteria of SSc (van den Hoogen *et al.*, 2013). Clinical and serological data were recorded for all patients (Table 1). One hundred and sixty four age- and sex-matched Greek-Cypriot healthy controls were recruited. Due to the small size of the population ($\sim$700,000 individuals), the number of recruited patients with SSc, was relatively small. Therefore, we used an increased number of control individuals per case with a ratio of 4:1, as suggested by Hong *et al.* (Hong and Park, 2012), to increase the statistical power of the study. This study was approved by the Cyprus National Bioethics Committee (ΕΕΒΚ/ΕΠ/2013/28, May 14, 2015 and ΕΕΒΚ/ΕΠ/2015/31, February 9, 2016) and conducted in accordance with the 1964 Declaration of Helsinki.

Genomic DNA extraction

DNA was extracted from whole blood samples using the DNA purification system Gentra Puregene Blood Core Kit C (Qiagen Sciences), according to the manufacturer's protocol.

Table 1. The Main Features of the Patients with Systemic Sclerosis and Healthy Control Participants

Trait	*Patients with SSc*	*Healthy controls*
Number	41	164
Sex (female: male)	(36:5)	(144:20)
Age (years, mean ± SD)[a]	60.10 ± 12.97	61.70 ± 11.56
Age of onset (years, mean ± SD)	49.05 ± 13.80	—
Subtype		—
lcSSc, *n* (%)	20 (48.78)	—
dcSSc, *n* (%)	21 (51.22)	—
Autoantibodies		—
ANA+, *n* (%)	39 (95.12)	—
ATA+, *n* (%)	14 (34.15)[b]	—
ACA+, *n* (%)	21 (51.22)[c]	—
Raynaud's phenomenon (%)	41 (100)	—
Smoking		—
Current smoker n (%)	6 (14.63)	—
Past smoker n (%)	8 (19.51)	—
Never n (%)	27 (65.85)	—

[a] $p = 0.47$ (patients age vs. control age).
[b] 13 (92.86%) out of 14 patients with dcSSc.
[c] 18 (85.71%) out of 21 patients with lcSSc.
ACA, anticentromere autoantibodies; ANA, antinuclear autoantibodies; ATA, antitopoisomerase autoantibodies; dcSSc, diffused cutaneous scleroderma; lcSSc, limited cutaneous scleroderma; SD, standard deviation.

SNPs selection

Eighteen SNPs previously associated with SSc in other populations, were selected (Appendix Table A1) based on our systematic review (Chairta *et al.*, 2017).

Genotyping

SNP genotyping was performed using either Sanger sequencing, restriction fragment length polymorphism (RFLP) (BsrBI and ApoI restriction enzymes [New England Biolabs]), or a SNaPshot Multiplex Minisequencing Assay (SNaPshot Multiplex Kit [Applied Biosystem, United Kingdom]). PCR and SNaPshot primers (Metabion International, Germany) were designed using the Primer3 (available upon request). Sanger sequencing and SNaPshot multiplex minisequencing samples were analyzed on an ABI 3130xl genetic analyzer (Applied Biosystems).

Statistical analysis

Quality control (QC) check was carried out for samples and SNPs. A chi-square test was used to evaluate the deviation from Hardy–Weinberg equilibrium (HWE) in healthy control samples. SNPs deviating from HWE were excluded from further consideration ($p < 0.05$ in controls). Minor allele frequency (MAF) for each SNP was calculated (MAF >0.01) in the control samples. The difference in frequency distribution of SNPs between patients and

healthy controls was examined using logistic regression analysis (log-additive model), having the common allele as reference.

A p-value of <0.05 was considered as nominally statistically significant, and corrected for multiple testing using Bonferroni correction for the SNPs that passed the HWE. Analyses were carried out with the R software. Odds ratio (OR) and 95% confidence intervals (CI) were calculated. Comparison of age distribution between case and control samples was performed using the independent samples t-test.

Results

Study participants

This study included 41 Greek-Cypriot unrelated patients with SSc (36 female and 5 male) with a mean ± standard deviation (SD) age of 60.10 ± 12.97 years, and 164 age- and sex-matched Greek-Cypriot unrelated healthy controls (144 female and twenty male) with a mean ± SD age of 61.70 ± 11.56. The main clinical and serological features of the patients are described in Table 1.

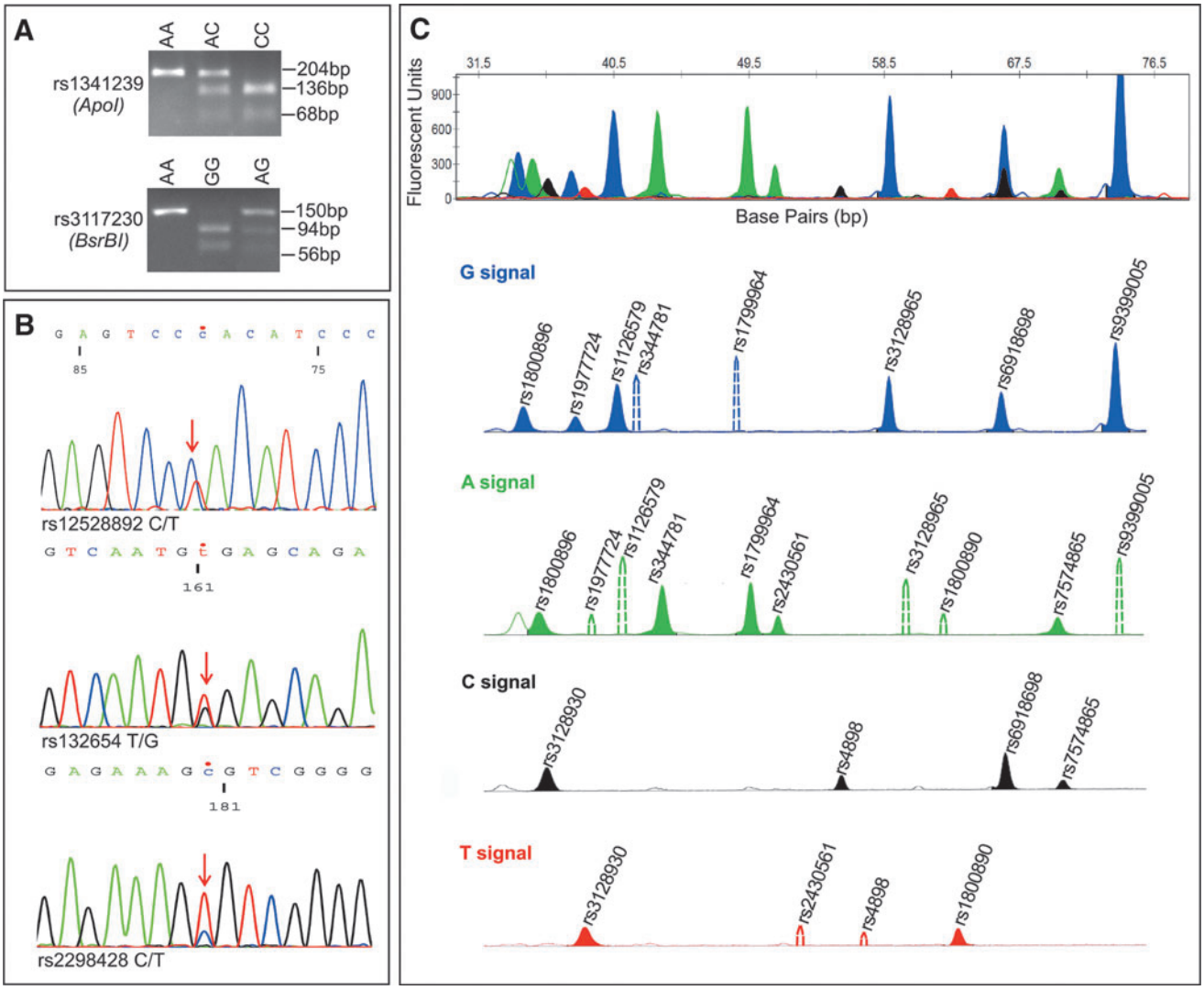

FIG. 1. Genotyping of eighteen SSc associated SNPs by RFLP, Sanger sequencing and multiplex SNaPshot minisequencing assay. **(A)** Genotyping of SNPs rs1341239 and rs3117230 using restriction enzymes *Apo*I and *Bsr*BI, respectively. **(B)** Sanger sequencing results show a heterozygous genotype of SNPs rs12528892, rs132654, and rs2298428, respectively. **(C)** The first electropherogram shows the detection of the thirteen SNPs in a random sample. Four separate electropherograms are presented, according to the color of peak (ddNTP extension). *Blue*, *green*, *black*, and *red-filled peaks* correspond to the fluorescence signal of G, A, C and T alleles of detected SNP, respectively. *Colored nonfilled solid peak* indicates nonspecific allele; this is not associated with the genotype. *Dotted peaks* represent the position of the alternative allele in the cases of homozygous SNP results. SNP genotype of the selected sample is the following: rs1800896 (*GA*), rs3128930 (*CT*), rs1799724 (*GG*), rs1126579 (*GG*), rs344781 (*AA*), rs1799964 (*AA*), rs2430561 (*AA*), rs4898 (*CC*), rs3128965 (*GG*), rs1800890 (*TT*), rs6918698 (*GC*), rs7574865 (*CA*), rs9399005 (*GG*). RFLP, restriction fragment length polymorphism; SNPs, single nucleotide polymorphisms; SSc, systemic sclerosis.

Table 2. Genotypes/Alleles Frequencies and Association of Selected Single Nucleotide Polymorphisms with Systemic Sclerosis Under Logistic Regression Analysis in the Greek-Cypriot Population (Patients with Systemic Sclerosis Versus Healthy Controls)

SNP	*Genotypes frequency,* n *(%)*			*Alleles frequency,* n *(%)*		*OR (95% CI)*[a]	p[a,b]
rs4898[c]	**T/T**	**T/C**	**C/C**	**T**	**C**	1.06 (0.65–1.74)	0.82
Controls (144)	58 (40.28)	59 (40.97)	27 (18.75)	175 (60.76)	113 (39.24)		
Cases (36)	13 (36.11)	17 (47.22)	6 (16.67)	43 (59.72)	29 (40.28)		
rs344781	**A/A**	**A/G**	**G/G**	**A**	**G**	0.97 (0.54–1.73)	0.91
Controls (164)	93 (56.71)	61 (37.20)	10 (6.10)	247 (75.30)	81 (24.70)		
Cases (41)	21 (51.22)	20 (48.78)	0 (0.00)	62 (75.61)	20 (24.39)		
rs1126579	**C/C**	**C/T**	**T/T**	**C**	**T**	1.07 (0.66–1.75)	0.78
Controls (164)	66 (40.24)	74 (45.12)	24 (14.63)	206 (62.80)	122 (37.20)		
Cases (41)	16 (39.02)	18 (43.90)	7 (17.07)	50 (60.98)	32 (39.02)		
rs1341239	**G/G**	**G/T**	**T/T**	**G**	**T**	1.01 (0.59–1.71)	0.98
Controls (164)	67 (40.85)	79 (48.17)	18 (10.98)	213 (64.94)	115 (35.06)		
Cases (41)	15 (36.59)	23 (56.10)	3 (7.32)	53 (64.63)	29 (35.37)		
rs1799724	**C/C**	**C/T**	**T/T**	**C**	**T**	1.22 (0.68–2.17)	0.51
Controls (164)	110 (67.07)	48 (29.27)	6 (3.66)	268 (81.71)	60 (18.29)		
Cases (41)	26 (63.41)	12 (29.27)	3 (7.32)	64 (78.05)	18 (21.95)		
rs1799964	**T/T**	**T/C**	**C/C**	**T**	**C**	0.94 (0.50–1.76)	0.84
Controls (164)	98 (59.76)	60 (36.59)	6 (3.66)	256 (78.05)	72 (21.95)		
Cases (41)	24 (58.54)	17 (41.46)	0 (0.00)	65 (79.27)	17 (20.73)		
rs1800890	**T/T**	**T/A**	**A/A**	**T**	**A**	0.82 (0.44–1.49)	0.51
Controls (164)	90 (54.88)	66 (40.24)	8 (4.88)	246 (75.00)	82 (25.00)		
Cases (41)	24 (58.54)	16 (39.02)	1 (2.44)	64 (78.05)	18 (21.95)		
rs1800896	**A/A**	**A/G**	**G/G**	**A**	**G**	1.25 (0.77–2.03)	0.35
Controls (164)	62 (37.80)	79 (48.17)	23 (14.02)	203 (61.89)	125 (38.11)		
Cases (41)	16 (39.02)	14 (34.15)	11 (26.83)	46 (56.10)	36 (43.90)		
rs2430561	**T/T**	**A/T**	**A/A**	**T**	**A**	1.00 (0.59–1.69)	1
Controls (164)	37 (22.56)	92 (56.10)	35 (21.34)	166 (50.61)	162 (49.39)		
Cases (41)	7 (17.07)	27 (65.85)	7 (17.07)	41 (50.00)	41 (50.00)		
rs3117230	**A/A**	**A/G**	**G/G**	**A**	**G**	2.27 (1.30–3.98)	**0.004**
Controls (164)	123 (75.00)	37 (22.56)	4 (2.44)	283 (86.28)	45 (13.72)		
Cases (41)	23 (56.10)	13 (31.71)	5 (12.20)	59 (71.95)	23 (28.05)		
rs3128930	**G/G**	**G/A**	**A/A**	**G**	**A**	2.08 (1.23–3.53)	**0.006**
Controls (164)	118 (71.95)	39 (23.78)	7 (4.27)	275 (83.84)	53 (16.16)		
Cases (41)	21 (51.22)	15 (36.59)	5 (12.20)	57 (69.51)	25 (30.49)		
rs3128965	**G/G**	**G/A**	**A/A**	**G**	**A**	1.97 (1.10–3.54)	**0.02**
Controls (164)	123 (75.00)	37 (22.56)	4 (2.44)	283 (86.28)	45 (13.72)		
Cases (41)	24 (58.54)	14 (34.15)	3 (7.32)	62 (75.61)	20 (24.39)		
rs6918698	**G/G**	**G/C**	**C/C**	**G**	**C**	0.88 (0.55–1.41)	0.59
Controls (164)	48 (29.27)	79 (48.17)	37 (22.56)	175 (53.35)	153 (46.65)		
Cases (41)	15 (36.59)	17 (41.46)	9 (21.95)	47 (57.32)	35 (42.68)		
rs7574865	**G/G**	**G/T**	**T/T**	**G**	**T**	1.35 (0.79–2.33)	0.27
Controls (164)	76 (46.34)	70 (42.68)	18 (10.98)	222 (67.68)	106 (32.32)		
Cases (41)	20 (48.78)	20 (48.78)	1 (2.44)	60 (73.17)	22 (26.83)		
rs9399005	**C/C**	**C/T**	**T/T**	**C**	**T**	0.76 (0.43–1.35)	0.35
Controls (164)	87 (53.05)	64 (39.02)	13 (7.93)	238 (72.56)	90 (27.44)		
Cases (41)	25 (60.98)	14 (34.15)	2 (4.88)	64 (78.05)	18 (21.95)		
rs12528892	**C/C**	**C/T**	**T/T**	**C**	**T**	0.80 (0.09–7.08)	0.84
Controls (164)	159 (96.95)	5 (3.05)	0 (0.00)	323 (98.48)	5 (1.52)		
Cases (41)	40 (97.56)	1 (2.44)	0 (0.00)	81 (98.78)	1 (1.22)		
rs131654	**T/T**	**T/G**	**G/G**	**T**	**G**	1.71 (0.99–2.96)	0.05
Controls (164)	93 (56.71)	63 (38.41)	8 (4.88)	249 (75.91)	79 (24.09)		
Cases (41)	17 (41.46)	20 (48.78)	4 (9.76)	54 (65.85)	28 (34.15)		
rs2298428	**C/C**	**C/T**	**T/T**	**C**	**T**	—	—
Controls (164)	104 (63.41)	32 (19.51)	28 (17.07)	240 (73.17)	88 (26.83)		
Cases (41)	30 (73.17)	7 (17.07)	4 (9.76)	67 (81.71)	15 (18.29)		

Bold in the last column indicates significant *p*-values.
[a]OR (CI 95%) and *p* value were calculated based on log-additive model (alleles).
[b]Nominal significance threshold = 0.05; Bonferroni corrected significance threshold = 0.003.
[c]Calculations for this SNP were performed using only the female patient genotypes since it is located on the X-chromosome.
CI, confidence intervals; OR, odds ratio; SNP, single nucleotide polymorphism.

Association studies and statistical analysis

Eighteen SNPs were selected and investigated using three different methodologies (Fig. 1). Seventeen out of eighteen SNPs passed QC checks. SNP rs2298428 was excluded as it deviated from the HWE (Appendix Table A1). Each SNP was analyzed for association with SSc and the relevant results are shown in Table 2. Associations of SSc with three SNPs (rs3117230, $p=0.004$; rs3128930, $p=0.006$; rs3128965, $p=0.02$) were observed in the Greek-Cypriot population at the $p<0.05$ level. However, none of these associations survives Bonferroni correction.

Discussion

A large number of studies support that HLA/non-HLA genetic variants and environmental factors play a key role in the triggering of SSc (Chairta *et al.*, 2017). Genetic heterogeneity has been observed among populations suggesting that investigation of SSc susceptibility in additional populations might provide a clearer understanding of disease etiology. The aim of this study was to evaluate SNP associations already discovered in other populations, within the Greek-Cypriots.

Forty-one Greek-Cypriot patients with SSc and 164 age- and sex-matched Greek-Cypriot healthy controls were included in this study. More females than males with SSc were recorded (7.2:1) and this difference follows the pattern also observed in other studies (Mayes, 2003; Chifflot *et al.*, 2008; Barnes and Mayes, 2012; Alba *et al.*, 2014; Ngo *et al.*, 2014). The mean age of onset of patients with SSc in our study was 49.05, within the ranges that have been reported worldwide (Alba *et al.*, 2014). Clinical and serological features did not differ from those of patients with SSc described in other studies.

Almost all Greek-Cypriot patients with SSc (95.12%) were positive for antinuclear autoantibodies (ANA). Similarly, Mierau *et al.* showed that 94.2% of patients with SSc in the German Network for Systemic Scleroderma were also positive for ANA (Mierau *et al.*, 2011). In our study lcSSc and dcSSc subgroups were mainly associated with anticentromere autoantibodies (ACA) and antitopoisomerase autoantibodies (ATA), respectively, in agreement with findings of other studies, where ACA has been strongly related with CREST syndrome (lcSSc) patients, while ATA was found in $\sim$40% of patients with dcSSc and <10% of patients with lcSSc (Tan *et al.*, 1980; Spencer-Green *et al.*, 1997; Ho and Reveille, 2003; Reveille and Solomon, 2003). Raynaud's phenomenon, which is one of the initial and obvious features in patients with SSc (Sunderkötter and Riemekasten, 2006), was observed in all patients of our study.

Through this study, we performed a replication/evaluation study because the number of recruited Greek-Cypriot patients with SSc was relatively small and thus statistical power for a discovery study could not be reached. A nominally significant association between SSc and three SNPs (rs3117230; rs3128930; rs3128965) has been detected under log-additive model (Table 2). Interestingly, these three SNPs are located on chromosome 6p in the region of *HLA-DPB1* and *HLA-DPB2*. These SNPs were significantly associated with SSc and the ATA+ SSc subgroup in the discovery phase of the Korean population study, but this association did not survive in the replication phase (Zhou *et al.*, 2009). Our results are consistent with the above study and may support the HLA region genetic susceptibility to SSc predisposition (Table 2 and Appendix Table A2).

Ten out of eighteen selected SNPs were previously reported to be significantly associated with SSc under log-additive model. In the current study, five (significantly associated: rs3128965, rs3117230, and rs3128930; nonsignificantly associated: rs1800896 and rs7574865) out of these ten SNPs have effects on the disease in the same direction as reported in previous studies (Appendix Table A2). Lack of association of the rest of the SNPs with SSc in the Greek-Cypriot population could be either attributed to genetic heterogeneity or small power of the study. In addition, the majority of investigated SNPs had similar frequencies with those reported in European populations in published studies/dbSNP (Appendix Tables A1 and A2).

SSc prevalence in Europe ranges from 31 to 277 cases/million individuals (Silman *et al.*, 1988; Allcock *et al.*, 2004; Le Guern *et al.*, 2004; Alamanos *et al.*, 2005; Arias-Nunez *et al.*, 2008; Lo Monaco *et al.*, 2011). To our knowledge, no epidemiological or genetic data on SSc in the Greek-Cypriot population have been previously reported. Based on epidemiological data reported in other populations, a relatively small number of SSc cases (21 to 193) is expected in our population. Thus, the number of patients analyzed through this study is a comparatively good representation of SSc in the Greek-Cypriot population. However, because a relatively small number of study subjects might lead to false results, we increased the number of controls per case (4:1) to limit this chance.

This is the first SSc susceptibility loci study in the Greek-Cypriot population and the majority of the investigated SNPs, do not confirm any statistically significant association with SSc in our population. Further investigation of the Greek-Cypriot population with a larger sample size may increase statistical power and enable identification of SSc susceptibility loci in this newly investigated population.

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Author Disclosure Statement

No competing financial interests exist.

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Appendix

Appendix Table A1. Details of Single Nucleotide Polymorphisms Selected for This Study

SNP	*Chromosomal position (GRCh38.p12)*	*Gene: consequence*	*Alleles*[a]	*MAF*[b]	*MAF in controls (current study)*	*HWE in controls (current study)*	*References*
rs4898[c]	chrX:47585586	*TIMP1:* Synonymous Variant *SYN1:* Intron Variant *MIR4769:* 2KB Upstream Variant	T/C	0.46	0.39	0.09	Indelicato *et al.* (2006), Skarmoutsou *et al.* (2012)
rs344781	chr19:43670636	*PLAUR:* 2KB Upstream Variant	A/G	0.25	0.25	1.00	Manetti *et al.* (2011)
rs1126579	chr2:218136011	*CXCR2:* 3 Prime UTR Variant	C/T	0.49	0.37	0.66	Renzoni *et al.* (2000), Salim *et al.* (2012)
rs1341239	chr6:22303975	*PRL:* 2KB Upstream Variant	G/T	0.35	0.35	0.46	Fojtíková *et al.* (2010)
rs1799724	chr6:31574705	*TNF:* 2KB Upstream Variant *LTA:* 500B Downstream Variant	C/T	0.09	0.18	0.79	Sato *et al.* (2004), Otieno *et al.* (2007)
rs1799964	chr6:31574531	*TNF:* 2KB Upstream Variant *LTA:* 500B Downstream Variant *LOC100287329:* 2KB Upstream Variant	T/C	0.21	0.22	0.39	Sato *et al.* (2004), Otieno *et al.* (2007)

(*Appendix continued* →)

Appendix Table A1. (Continued)

SNP	*Chromosomal position (GRCh38.p12)*	*Gene: consequence*	*Alleles*[a]	*MAF*[b]	*MAF in controls (current study)*	*HWE in controls (current study)*	*References*
rs1800890	chr1:206776020	*IL19:* Intron Variant	T/A	0.37	0.25	0.35	Hudson *et al.* (2005), Peng *et al.* (2012b)
rs1800896	chr1:206773552	*IL19:* Intron Variant *IL10:* 2KB Upstream Variant	A/G	0.45	0.38	0.79	Ates *et al.* (2008), Salim *et al.* (2013)
rs2430561	chr12:68158742	*IFNG:* Intron Variant	T/A	0.46	0.49	0.12	Wastowski *et al.* (2009)
rs3117230	chr6:33107858	*HLA-DPB1*: Downstream Variant	A/G	0.23	0.14	0.55	Zhou *et al.* (2009)
rs3128930	chr6:33107889	*HLA-DPB1*: Downstream Variant	G/A	0.26	0.16	0.12	Zhou *et al.* (2009)
rs3128965	chr6:33088122	*HLA-DPB1:* 3 Prime UTR Variant	G/A	0.19	0.14	0.55	Zhou *et al.* (2009)
rs6918698	chr6:131952117	*CCN2:* 2KB Upstream Variant	G/C	0.49	0.47	0.68	Fonseca *et al.* (2007), Kawaguchi *et al.* (2009)
rs7574865	chr2:191099907	*STAT4:* Intron Variant	G/T	0.23	0.32	0.76	Dieude *et al.* (2009), Gourh *et al.* (2009), Rueda *et al.* (2009), Tsuchiya *et al.* (2009), Allanore *et al.* (2011), Liang *et al.* (2012), Peng *et al.* (2012a), Yi *et al.* (2013), Zheng *et al.* (2013), Zochling *et al.* (2014), Xu *et al.* (2016)
rs9399005	chr6:131947824	*CCN2:* 500B Downstream Variant	C/T	0.30	0.27	0.80	Granel *et al.* (2010)
rs12528892	chr6:32725729	*HLA-DQA2:*Upstream variant	C/T	0.07	0.02	0.84	Mayes *et al.* (2014)
rs131654	chr22:21562901	*UBE2L3:* Intron Variant	T/G	0.33	0.24	0.52	Hasebe *et al.* (2012)
rs2298428	chr22:21628603	*YDJC:* Missense Variant	C/T	0.18	0.27	0.00	Hasebe *et al.* (2012)

[a]Major/Minor allele based on the current and published studies.
[b]MAF of European population submitted in 1000 Genome Project (dbSNP).
[c]Calculations for this SNP in the current study were performed using only the female patient genotypes since it is located on the X-chromosome.
HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

(*Appendix continues* →)

Appendix Table A2. Previously Reported Single Nucleotide Polymorphism Associations Based on Systemic Sclerosis Patients Versus Healthy Controls

SNP	*Alleles*	*Cases alleles n (%)*	*Controls alleles n (%)*	*OR (95% CI)*	p	*Cases/ controls*	*Population*	References
rs4898[a]	T	269 (65.29)	180 (57.69)	1	—	206/156	Italian	Skarmoutsou *et al.* (2012)
	C	143 (34.71)	132 (42.31)	0.72 (0.53–0.98)	0.04			
rs344781	G	210 (27)	180 (23)	1.29 (1.03–1.63)	0.03	388/391	Italian (Caucasian)	Manetti *et al.* (2011)
	A	566 (73)	602 (77)	—				
	G	366 (25)	256 (21)	1.22 (1.02–1.47)	0.03	732/607	Italian and French (Caucasian)	
	A	1098 (75)	959 (79)	—	—			
rs1126579	T	287 (56)	341 (44)	NA	0.002	256/388	United Kingdom (Caucasian)	Renzoni *et al.* (2000)
	C	225 (44)	435 (56)	—				
rs1800890	T vs. A	—	—	0.75 (0.61–0.93)	NA	382/1125	Meta-analysis (4 studies)	Peng *et al.* (2012b)
rs1800896	G	45 (50)	78 (26)	2.85 (1.74–4.63)	<0.000	45/150	Turkish	Ates *et al.* (2008)
	A	45 (50)	222 (74)	—	—			
rs3117230	G	48 (18)	102 (9.2)	2.20 (1.50–3.22)	3.52E-05	133/557	Discovery, Koreans	Zhou *et al.* (2009)
	A	218 (82)	1012 (90.8)	—				
rs3128930	A	90 (34)	167 (15)	3.0 (2.20–4.10)	8.16E-13	133/557	Discovery, Koreans	Zhou *et al.* (2009)
	G	176 (66)	947 (85)	—	—			
rs3128965	A	48 (18)	104 (9.3)	2.18 (1.49–3.18)	4.47E-05	133/557	Discovery, Koreans	Zhou *et al.* (2009)
	G	218 (82)	1114 (90.7)	—	—			
rs6918698	G	435 (55)	241 (45)	1.5 (1.2–1.9)	<0.001	395/269	Asian (Japanese)	Kawaguchi *et al.* (2009)
	C	355 (45)	297 (55)					
rs7574865	T	218 (27.1)	220 (22.9)	1.26 (1.01–1.56)	0.039	402/481	Discovery, French	Dieude *et al.* (2009)
	G	586 (72.9)	742 (77.1)	—	—			
	T	212 (26.6)	206 (21.3)	1.35 (1.07–1.66)	0.0099	399/483	Replication, French	
	G	586 (73.4)	760 (78.7)	—	—			
	T	429 (26.8)	426 (22.1)	1.29 (1.11–1.51)	0.001	801/964	Combination, French	
	G	1173 (73.2)	1502 (77.9)	—	—			
	T	458 (26)	213 (21)	1.31 (NA)	0.01	880/507	North American	Gourh *et al.* (2009)
	G	1302 (74)	801 (79)	—	—			
	T	757 (27)	315 (22.5)	1.26 (1.1–1.5)	0.004	1402/698	North American	
	G	2047 (73)	1082 (77.5)	—	—			
	T	231 (41)	401 (34)	1.35 (1.10–1.66)	0.023	282/590	Japanese	Tsuchiya *et al.* (2009)
	G	333 (59)	779 (66)	—	—			
	T	307 (27.2)	778 (21.9)	1.33 (1.14–1.55)	2.50E-04	564/1776	Discovery, French	Allanore *et al.* (2011)
	G	821 (72.8)	2774 (78.1)					
	T	976 (29)	1727 (22)	1.40 (1.26–1.56	1.9E-10	1682/3926	Replication (French, Italians, German & Eastern European)	
	G	2388 (71)	6124 (78)	—	—			
	T vs. G	—	—	0.72 (0.66–0.79)	0.00	—	Meta-analysis (8 studies)	Peng *et al.* (2012a)
	T vs. G	—	—	1.34 (1.25–1.44)	<0.00001	—	Meta-analysis (11 studies)	Liang *et al.* (2012)
	T allele	—	—	1.38 (1.27–1.50)	<1.44E-14	—	Meta-analysis (8 studies)	Zheng *et al.* (2013)
	G	565 (62.4)	744 (69.7)	0.72 (0.48–1.09)	0.1	453/534	Han Chinese	Yi *et al.* (2013)
	T	341 (37.6)	324 (30.3)	1.58 (1.22–2.05)	0.00041			
	T	278 (28.6)	2006 (22.5)	1.35	0.00012	486/4458	Discovery, Australian SSc, British controls	Zochling *et al.* (2014)
	G	694 (71.40)	6909 (77.5)	—	—			
	T	NA	NA	NA	5.7 E-5	1319/6396	Combination, Australian SSc, British controls	
	G	NA	NA	—	—			
	T vs. G	—	—	1.37 (1.27–1.48)	<0.00001	—	Meta-analysis (4 studies)	Xu *et al.* (2016)

[a]Calculations for this SNP were performed in females as it is located in X-chromosome.
CI, confidence intervals; OR, odds ratio; SSc, systemic sclerosis.