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 (~700,000 individuals), the number of recruited patients with SSc was relatively small. Therefore, we used an increased number of control individuals per case for all cases (Table 1), to increase the statistical power of the study. This study was approved by the Cyprus National Bioethics Committee (EEBK/EIT/2013/28, May 14, 2015 and EEBK/EIT/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.

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).

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 controls was assessed using a chi-square test.
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 statisti-
cally 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 minise-
quencing assay. (A) Genotyping of SNPs rs1341239 and rs3117230 using restriction enzymes Apol and BsrBI, 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 electrophere-
grams are presented, according to the color of peak (ddNTP extension). Blue, green, black, and red-filled peaks corre-
spond to the fluorescence signal of G, A, C and T alleles of detected SNP, respectively. Colored nonfilled solid peak
depicts 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) | p<sup>a,b</sup> |
|-------|-----------------------------|--------------------------|-------------|-----------------|
| rs4898<sup>c</sup> | T/T 58 (40.28) | T/C 59 (40.97) | C/C 27 (18.75) | 1.06 (0.65–1.74) | 0.82 |
| Controls (144) | | | | |
| Cases (36) | A/A 13 (36.11) | G/G 17 (47.22) | C/C 7 (19.44) | 0.97 (0.54–1.73) | 0.91 |
| rs344781 | A/G 93 (57.61) | T/T 61 (37.20) | G/G 10 (6.19) | 0.82 (0.50–1.38) | 0.35 |
| Controls (164) | | | | |
| Cases (41) | C/C 21 (51.22) | C/T 20 (48.78) | T/T 0 (0.00) | 1.00 (0.59–1.69) | 1.00 |

**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 ~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; Alcock 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 | MAF in controls (current study) | MAF in controls (current study) | HWE in controls (current study) | References |
|---------|-----------------------------------|-------------------|---------|---------------------------------|---------------------------------|---------------------------------|------------|
| rs4898  | chrX:47585586                      | **TIMP1**: Synonymous 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 | C/T     | 0.09                            | 0.18                            | 0.79                            | Sato et al. (2004), Otieno et al. (2007) |
| rs1799964| chr6:31574531                      | **TNF**: 2KB Upstream Variant | T/C     | 0.21                            | 0.22                            | 0.39                            | Sato et al. (2004), Otieno et al. (2007) |

(Appendix continued →)
| SNP        | Chromosomal position (GRCh38.p12) | Gene: consequence | Alleles | MAF<sup>b</sup> in controls (current study) | MAF<sup>b</sup> in controls (current study) | References |
|------------|----------------------------------|-------------------|---------|---------------------------------------------|---------------------------------------------|------------|
| rs1800890  | chr1:206776020                   | *IL19*: Intron Variant | T/A     | 0.37                                        | 0.25                                        | 0.35       |
| rs1800896  | chr1:206773552                   | *IL19*: Intron Variant | A/G     | 0.45                                        | 0.38                                        | 0.79       |
| rs2430561  | chr12:68158742                   | *IFNG*: Intron Variant | T/A     | 0.46                                        | 0.49                                        | 0.12       |
| rs3117230  | chr6:33107858                    | *HLA-DPB1*: Downstream Variant | T/A     | 0.23                                        | 0.14                                        | 0.55       |
| rs3128930  | chr6:33107889                    | *HLA-DPB1*: Downstream Variant | G/A     | 0.26                                        | 0.16                                        | 0.12       |
| rs3128965  | chr6:33088122                    | *HLA-DPB1*: 3 Prime UTR Variant | G/A     | 0.19                                        | 0.14                                        | 0.55       |
| rs6918698  | chr6:131952117                   | *CCN2*: 2KB Upstream Variant | G/C     | 0.49                                        | 0.47                                        | 0.68       |
| rs7574865  | chr2:191099907                   | *STAT4*: Intron Variant | G/T     | 0.23                                        | 0.32                                        | 0.76       |
| rs9399005  | chr6:131947824                   | *CCN2*: 500B Downstream Variant | C/T     | 0.30                                        | 0.27                                        | 0.80       |
| rs12528892 | chr6:32725729                    | *HLA-DQA2*: Upstream variant | C/T     | 0.07                                        | 0.02                                        | 0.84       |
| rs131654   | chr22:21562901                   | *UBE2L3*: Intron Variant | T/G     | 0.33                                        | 0.24                                        | 0.52       |
| rs2298428  | chr22:21628603                   | *YDJC*: Missense Variant | C/T     | 0.18                                        | 0.27                                        | 0.00       |

<sup>a</sup>Major/Minor allele based on the current and published studies.

<sup>b</sup>MAF of European population submitted in 1000 Genome Project (dbSNP).

<sup>c</sup>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.
| SNP     | Alleles | Cases alleles n (%) | Controls alleles n (%) | OR (95% CI) | p     | Cases/controls | Population          | References          |
|---------|---------|---------------------|------------------------|-------------|-------|----------------|----------------------|---------------------|
| rs4898<sup>a</sup> | T       | 269 (65.29)         | 180 (57.69)            | 1           | —     | 206/156        | Italian              | Skarmoutsou et al. (2012) |
|         | G       | 143 (34.71)         | 132 (42.31)            | 0.72 (0.53–0.98) | 0.04  |               |                      | Manetti et al. (2011)  |
|         | C       | 132 (42.31)         | 132 (42.31)            | 1.29 (1.03–1.63) | 0.03  |               | Italian and French (Caucasian) |                       |
|         | A       | 602 (77)            | 602 (77)               | —           | —     | 732/607        |                       | Renzoni et al. (2000) |
| rs1126579 | T      | 287 (56)            | 341 (44)               | NA          | 0.002 | 256/388        | United Kingdom (Caucasian) |                       |
|         | C      | 225 (44)            | 435 (56)               | —           | —     |               |                      | 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)    |
| rs3117230 | G      | 566 (73)            | 602 (77)               | —           | —     | 732/607        |                       | Zhou et al. (2009)    |
| rs3128930 | G      | 1098 (75)           | 959 (79)               | —           | —     | 732/607        |                       | Zhou et al. (2009)    |
| rs3128965 | A      | 48 (18)             | 104 (9.3)              | 2.18 (1.49–3.18) | 4.47E-05 | 133/557 Discovery, Koreans | Zhou et al. (2009)    |
| rs754865  | T       | 218 (27.1)          | 220 (22.9)             | 1.26 (1.01–1.56) | 0.039 | 402/481        | Discovery, French     | Dieder et al. (2009)  |
|         | G       | 586 (72.9)          | 742 (77.1)             | —           | —     | 398/493        | Replication, French   |                     |
|         | T       | 212 (26.6)          | 206 (21.3)             | 1.35 (1.07–1.66) | 0.0099 |               | French               |                     |
|         | G       | 586 (73.4)          | 760 (78.7)             | —           | —     | 801/964        | Combination, French   |                     |
|         | T       | 429 (26.8)          | 426 (22.1)             | 1.29 (1.11–1.51) | 0.001 |               | French               |                     |
|         | G       | 1173 (73.2)         | 1502 (77.9)            | —           | —     | 880/507 North |                       | Gourh et al. (2009)   |
|         | T       | 458 (26)            | 213 (21)               | 1.31 (NA)   | 0.01  | 132/557 North |                       |                     |
|         | G       | 1302 (74)           | 801 (79)               | —           | —     | 1402/698 North |                       |                     |
|         | T       | 757 (27)            | 315 (22.5)             | 1.26 (1.1–1.5) | 0.004 |               | North American       |                     |
|         | G       | 2047 (73)           | 1082 (77.5)            | —           | —     | 282/590 Japanese |                       | Tsuchiya et al. (2009) |
|         | T       | 231 (41)            | 401 (34)               | 1.35 (1.10–1.66) | 0.023 |               |                      |                     |
|         | G       | 333 (59)            | 779 (66)               | —           | —     | 564/1776 Discovery, French |  |                     |
|         | T       | 307 (27.2)          | 778 (21.9)             | 1.33 (1.14–1.55) | 2.50E-04 | 1682/3926 Replication, French, Italian, German & Eastern European |  |                     |
|         | G       | 821 (72.8)          | 2774 (78.1)            | —           | —     | 1682/3926 Replication, French, Italian, German & Eastern European |  |                     |
|         | T       | 976 (29)            | 1727 (22)              | 1.40 (1.26–1.56) | 1.9E-10 | 1682/3926 Replication, French, Italian, German & Eastern European |  |                     |
|         | G       | 2388 (71)           | 6124 (78)              | —           | —     | 1682/3926 Replication, French, Italian, German & Eastern European |  |                     |

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) <0.000001 — 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 T vs. G — — 1.37 (1.27–1.48) <0.00001 — Meta-analysis (4 studies) Xu et al. (2016)  

<sup>a</sup>Calculations for this SNP were performed in females as it is located in X-chromosome.

CI, confidence intervals; OR, odds ratio; SSc, systemic sclerosis.