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A 3-factor epistatic model predicts digital ulcers in Italian scleroderma patients

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ABSTRACT

Background: The genetic background may predispose systemic sclerosis (SSc) patients to the development of digital ulcers (DUs).
Methods: Twenty-two functional cytokine single nucleotide polymorphisms (SNPs) and 3 HLA class I and II antigens were typed at the genomic level by polymerase chain reaction in 200 Italian SSc patients. Associations with DUs were sought by parametric models and with the Multifactor Dimensionality Reduction (MDR) algorithm to depict the presence of epistasis. Biological models consistent with MDR results were built by means of Petri nets to describe the metabolic significance of our findings.
Results: On the exploratory analysis, the diffuse cutaneous subset (dcSSc) was the only single factor statistically associated with DUs ($p = 0.045$, ns after Bonferroni correction). Gene–gene analysis showed that a 3-factor model comprising the IL-6 C-174G, the IL-2 G-330T SNPs and the HLA-B*3501 allele was predictive for the occurrence of DUs in our population (testing accuracy = 66.9%; $p < 0.0001$, permutation testing).
Conclusion: Biological interpretation via Petri net showed that IL-6 is a key factor in determining DUs occurrence and that this cytokines may synergise with HLA-B*3501 to determine DUs onset. Owing to the limited number of patients included in the study, future research are needed to replicate our statistical findings as well as to better determine their functional meaning.

1. Introduction

Digital ulcers (DUs) are a frequent complication of systemic sclerosis (SSc) that may occur in up to 50% of patients during their disease history [1–4]. The occurrence of DUs has been associated with several clinical features of the disease in different populations, such as a younger age at onset of the disease [2–6], the male gender [2], the diffuse cutaneous subset (dcSSc) [2,6,7], the anti-topoisomerase I antibody [2,6], the presence of pulmonary hypertension [6], the presence of systemic inflammation [6,7] or a delay in introducing vasodilating therapy with calcium channel-blockers or iloprost (4). Whatever the association with other clinical features, ischemic DUs are thought to be the consequence of endothelial injury and small-vessel vasculopathy [8]. Yet, autoimmunity with involvement of T lymphocytes and mcrophages and the local release of cytokines with pro-inflammatory, as well as chemoattractant, properties are also relevant in the early onset of endothelial injury and thus in the development of DUs. Indeed, it has been shown that interleukin-6 (IL-6) plasma levels are increased in patients with SSc and DUs [7], and that this group of patients has increased plasma soluble CD40 ligand concentrations [9], reflecting both an inflammatory state and lymphocyte T activation. Also, endothelin-1 (ET-1) blockade by bosentan was shown effective in preventing the recurrence of new DUs in SSc, confirming, in this context, a pathophysiological role for ET-1 which is actively released by the damaged endothelium [10,11].

The secretion and/or bioavailability of cytokines with immunomodulatory, pro-fibrotic or pro-inflammatory function is regulated at the genetic level [12–16], hence it could be hypothesized that single nucleotide polymorphisms (SNPs) in or near cytokine genes may be relevant in determining vasculopathy and thus the appraisal of DUs in SSc patients. This hypothesis was verified in the present study as part of a project aimed at finding a possible role for genetic variants with well-known regulatory functions on cytokine production [17] and SSc or its clinical expressions [18–22]. Besides the possible association with DU and 22 cytokine gene SNPs, we also sought a possible association with the human leukocyte antigen system (HLA) allele, HLA-B*3501, that was previously shown to regulate *in vitro* ET-1 secretion [23] and endothelial cell apoptosis [24], and that was also associated with other vascular complications of SSc, such as isolated pulmonary hypertension [25]. In light of the multifactorial pathogenesis of SSc, we searched for high-order non-linear gene–gene interactions (epistasis) and built models of biochemical and

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physiological networks that are consistent with high-dimensional models of disease susceptibility to better explain these associations.

2. Methods

2.1. Patients selection

The patients used in this study consisted of two-hundred unrelated Italian SSc patients who were referred to our outpatient clinic. They provided written consent to have their DNA collected as well as their clinical data recorded and utilised for medical research. The vast majority of the patients ($n=181$, 91%) fulfilled the classification criteria proposed by the American College of Rheumatology [26], yet we also considered patients ($n=19$, 9%) with definite SSc who do not fulfil these criteria [1]. Patients were categorised as having Early SSc, the "sine scleroderma," the limited cutaneous (lcSSc) or the dcSSc subset [1,27]. History of DUs was ascertained by reviewing the patients' medical records. Disease onset was determined by the patient's recall of the first non-Raynaud symptom clearly attributable to scleroderma [28] and were categorised into 3 groups according to the age at onset: ≤30 years, 31–50 years, >50 years. Restrictive lung disease was defined as a forced vital capacity (FVC) ≤70% of predicted, with a normal Tiffenau's index [1]; diffusing capacity for carbon monoxide (DLco) impairment was considered for values ≤70% of predicted [1]. Pulmonary hypertension was defined as a right-ventricular systolic pressure (RVSP) ≥40 mm Hg and was confirmed by right-heart catheterisation. Antinuclear antibodies (ANA) were determined by indirect immunofluorescence on Hep_2 cells (Kallestad, Chaska, MN) using a standardised technique [29]; extractable nuclear antigens (ENAs) were determined by a commercial enzyme-linked immunoassay (ELISA) (Diamedix, Miami, FL).

2.2. Cytokine SNP determination

Blood samples were collected in citrate and DNA was extracted using the DNA Isolation Kit For Mammalian Blood (Roche Diagnostics, Indianapolis, IN). Genotyping was performed by polymerase chain reaction (PCR) with sequence-specific primers (PCR-SSP) according to the 13th International Histocompatibility Workshop recommendations, and by using the commercial Cytokine kit provided by the University Clinic of Heidelberg (CTS-PCR-SSP TRAY, from the Institute of Immunology, Department of Transplantation Immunology, University of Heidelberg, Heidelberg, Germany) as previously described [21]. Overall, the following 22 interleukin (IL) SNPs were analysed: IL-1 alpha (IL-1α) C-889T, IL-1 beta (IL-1β) C-511T and IL-1β C+3962T, IL-1 receptor (IL-1R) Cpst1970T, IL-1 receptor antagonist (IL-1Ra) Cmspal11100T, IL-2 G-330T, IL-2 G+160T, IL-4 G-1098T, IL-4 C-590T, IL-4 C-33T, IL-4 receptor antagonist alpha (IL-4Rα) A+1902G, IL-6 C-174G, IL-6 Ant565G, IL-10 A-1082G, IL-10 C-819T, IL-10 A-590C, IL-12 A-1188C, transforming growth factor beta (TGF-β1) T/C codon 10, TGF-β1 G/C codon 25, interferon gamma (IFNγ) AUTR5644T, tumor necrosis factor alpha (TNFα) A-308G, TNFα A-238G.

Typing results for the IL-4 G-1098T, IL-4 C-590T, IL-4 C-33T did not meet the quality requirements for interpretation (e.g. because of unequal or weak amplification results) and were therefore excluded from analysis.

2.3. HLA genotyping

The following HLA class I and II antigens were considered for analysis: HLA*B3501, that was previously associated with an increased production of ET-1 or cell apoptosis *in vitro* and *ex vivo* [23,24], HLA-DR*11 and HLA-DR*07,which were reported to be associated with SSc in Italian subjects [30].

HLA class I and class II antigens were typed at genomic level by polymerase chain reaction–sequence-specific oligonucleotide probes as previously described elsewhere [31,32]. Products of the PCR reaction were purified using Microcom columns (Amicon, Beverly, MA, USA), while products of the sequencing reaction were purified using CentriSep Spin Columns (Applied Biosystems, Monza, Italy). The sequencing of exons was done with BigDye Primers (Applied Biosystems). The sequencing reaction products were resuspended in 6 ml of formamide buffer (5:1), from which 1.75 ml was taken to be electrophoresed on 5% polyacrylamide (Applied Biosystems) 6 M urea gels with an ABI PRISM 377 DNA sequencer. DRB1 locus was typed by BigDye Terminator SBT Typing Kit (Applied Biosystems).

2.4. Statistical analysis

The distribution of genotypes was tested for Hardy–Weinberg equilibrium (HWE) with the goodness-of-fit χ^2 test at a significance level of 0.05. The distribution of clinical features, cytokine SNP variations, and of HLA alleles in SSc patients with or without DU was tested by the χ^2 test or Fisher's exact test when necessary. Variation in a particular SNP was considered to be associated with the endpoint at a significance level of 0.05 after Bonferroni correction.

2.5. MDR

The evaluation of gene–gene interactions was performed using the Multifactor Dimensionality Reduction (MDR) algorithm [33,34]. Using the MDR algorithm we constructed a series of combinations of two-to-four variables and then used a naïve Bayes classifier in the context of 10-fold cross-validation to estimate the testing accuracy (TA) of each best two-to-four factor model. A single best model was selected that maximized the TA. This is the model that is most likely to generalize to independent datasets. Statistical significance of the final model was evaluated using a 1000-fold permutation test to compare observed testing accuracies with those expected under the null hypothesis of no association. Permutation testing corrects for multiple testing by repeating the entire analysis on 10,000 datasets that are

Table 1
Clinical and demographic characteristics. Demographic and clinical characteristics of 200 Italian systemic sclerosis (SSc) patients with or without a personal history of digital ulcers (DU). lcSSc, limited SSc; dcSSc, diffuse SSc; ANA, antinuclear antibodies; ACA, anticentromere antibodies; Scl70, anti-topoisomerase I antibody; FVC, forced vital capacity; DLco, diffusing capacity for carbon monoxide; PAH, pulmonary hypertension (right-heart catheterisation).

Variable	SSc ($n=200$)	DU ($n=111$)	No DU ($n=89$)
Subset, n (%)			
Early SSc	14 (7)	5 (4.5)	9 (10.1)
Sine SSc	4 (2)	2 (1.8)	2 (2.3)
lcSSc	137 (68.5)	73 (65.8)	64 (71.9)
dcSSc*	45 (22.5)	31 (27.9)	14 (15.7)
Autoantibodies, n (%)			
ANA	195 (97.5)	107 (96)	88 (99)
ACA	82 (42)	44 (40)	40 (45)
Scl70	81 (40)	44 (40)	37 (42)
Females, n (%)	179 (89.5)	97 (87)	82 (92)
Age at onset, n (%)			
≤30 years	17 (8.5)	15 (13.5)	12 (13.5)
31–50 years	70 (35)	41 (36.9)	29 (32.6)
>50 years	97 (48.5)	49 (49.6)	48 (53.9)
Disease duration, years	14±3.1	14.8±2.7	13.5±3.6
FVC≤70% predicted, n (%)	49 (24.5)	29 (26)	20 (22)
DLco≤70% predicted, n (%)	79 (39.5)	46 (41)	33 (37)
PAH, n (%)	17 (8.5)	11 (9.9)	6 (6.7)

* $p=0.045$ vs the other disease subtypes (not significant after Bonferroni correction).