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Lack of association of the *PTPN22* gene polymorphism R620W with systemic sclerosis

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ABSTRACT

Objective. *It has recently been reported that some autoimmune diseases seem to be associated with a functional polymorphism in* PTPN22*, a gene which encodes a phosphatase known to be important in T-cell signaling. The aim of our study was to check for the prevalence of the* PTPN22 *R620W polymorphism in patients with systemic sclerosis.*

Methods. *DNA samples from 54 systemic sclerosis patients and 55 healthy controls were obtained from peripheral blood and genotyping was performed by means of a restriction fragment length polymorphism analysis of PCR products (RFLP-PCR).*

Results. *Allele frequency for the T allele was slightly higher in the patients group (0.074 versus 0.055). Eight out of the 54 systemic sclerosis patients (14.8 %) were heterozygous for this single nucleotide polymorphism whereas the CT genotype was found in 6 out of the 55 controls (10.9%). Nevertheless, the difference did not reach statistical significance (p = 0.542). Neither certain antibodies linked to systemic sclerosis (anti-centromere and anti-topoisomerase I antibodies) nor any particular clinical involvement were associated with the polymorphism.*

Conclusions. *This particular single nucleotide polymorphism of* PTPN22 *does not seem to be associated with systemic sclerosis.*

Introduction

Systemic sclerosis (SSc) is a multisystemic inflammatory disorder characterized by cutaneous and visceral fibrotic changes (1). An overproduction and accumulation of collagen in skin and viscera is the major pathological feature and microvasculature abnormalities are also frequently found (2). It is considered an autoimmune disease and anti-centromere and anti-topoisomerase I antibodies are frequently reported in different subsets of SSc (3, 4). Furthermore, it has become clear that the activated cellular-immune system plays a central role in the pathogenesis of SSc. Early on the presentation of the disease, macrophage infiltrates have been detected in biopsies from affected skin (5). These cells are $CD14^+$ and express Class II MHC, which suggest that they are activated. Prior activation of peripheral blood T cells in SSc is also evident by the increased expression of high affinity IL-2R and the fact that sera from SSc patients contain a three-fold higher level of serum IL-2. An uncontrolled activation state could be responsible for the release of different cytokines that in turn would initiate and/or perpetuate the fibrotic process as well as the endothelial and vascular alterations; the fibroblast proliferation and collagen production as well as various endothelial cell functions are indeed modulated by them (6, 7).

Protein tyrosine phosphatases (PTPs) are involved in T-cell activation; in this case, their mechanism of action consists on the dephosphorilation and inactivation of the T-cell antigen receptor (TCR)-associated kinases and their substrates. Among PTPs, the protein (lymphoid-specific phosphatase, LYP) encoded by the *PTPN22* gene has recently been found to play an important role as a negative regulator of T-cell activation. In fact, its deficiency induced by RNA interference (8) or gene targeting (9) causes marked increases in TCR signaling. LYP interacts through its most N-terminal proline-rich domain (P1) with the SH3 domain of the Csk kinase, an important suppressor of the Src family kinases (such as Lck and Fyn), which mediate TCR signaling. *In vitro* experiments have shown that the substitution of a highly conserved arginine by tryptophan (R620W) in the P1 domain avoids such an interaction between LYP and Csk (8, 10); consequently, the suppression of the T-cell activation does not take place. This substitution is due to a single nucleotide polymorphism (SNP) of *PTPN22* (1858C → T; National Center for Biotechnology Information dbSNP no. rs2476601).

Lack of suppression of T-cell activation may obviously lead to a hyper-reactive state that could be associated to the susceptibility to autoimmune disease. The first to make such an assumption and to confirm the potential role of the

R620W polymorphism were Bottini *et al.* (10). They reported the association of the SNP with type 1 diabetes mellitus (T1D). Later on, other groups have corroborated not just the increased frequency of the CT and TT genotypes among TD1 patients, but also in other autoimmune diseases such as rheumatoid arthritis (RA) (8), systemic lupus erythematosus (SLE) (11), and Graves' disease (12), among others. In some cases, the presence of the minor allele seems to be linked to the severity of the disease as in rheumatoid arthritis (RA) (13), and a dose effect has also been established in both SLE and RA, with the risk conferred by the homozygous TT genotype being twice that conferred by heterozygosity (11, 14). All these studies prompt us, therefore, to think about the possibility of this SNP being linked either to autoimmunity in general or to some autoimmune illnesses in particular. In order to ascertain the true role of this mutation in SSc, we evaluated its presence in a Spanish Caucasian population.

Patients and methods

Patients

A total of 54 SSc women and 55 healthy control women from the Internal Medicine Department of Hospital Vall d'Hebron were enrolled. Patients were classified according to LeRoy's criteria (1). Data on immunological findings [anti-centromere (determined by an immunofluorescence technique) and anti-topoisomerase I antibodies (determined by immunoblotting)] and several clinical manifestations (pulmonary arterial hypertension, interstitial lung disease, scleroderma renal crisis, and cardiac involvement) were available for most of the patients. Criteria for evaluating such manifestations have been previously detailed by us (15). Written informed consent was obtained for each participant and all the procedures followed were in accordance with the standards of our Hospital's committee.

Genotyping analysis

Genomic DNA was prepared from whole blood using the QIAamp DNA Blood Maxi Kit (QIAGEN GmbH, Hilden, Germany) and a 215 bp-polymerase chain reaction (PCR) fragment was generated. GeneCraft (Heidelberg, Germany) supplied all the reagents but the primers, which were purchased from Invitrogen Life Technologies (Paisley, Scotland). Measures were taken to prevent contamination and a negative control (water) was included in each run. Besides the DNA, each reaction contained 0.2 Mm dNTPs, 2.5 mM $MgCl_2$, 1X Taq polymerase buffer, 0.5 units of Taq DNA polymerase, and 10 pmoles of primers P1 and P2 (P1: 5'-TCACCAGCTTCCTCAACCACA-3'; P2: 5'-GATAATGTTGCTTCAACGGAATTTA-3'). Twenty-five-microliter reactions were carried out in a Whatman Biometra Tgradient thermocycler (Goettingen, Germany) under the following conditions: denaturation at 95ºC for 5 min.; thirty-five amplification cycles consisting of 30 sec. at 95ºC, 30 sec. at 60ºC, and 30 sec. at 66ºC; and seven additional minutes at 68ºC.

The newly made 215 bp DNA fragment was then digested with *Xcm* I under the following conditions: 13.1 µl of the reaction were incubated with 2 units of *Xcm* I (New England Biolabs Inc., Beverly, MA, USA) along with its corresponding buffer. The base change creates a new cleavage site for this enzyme. Thus, if the mutation is present, two digested products are obtained, their sizes being of 170 bp and 45 bp (see Fig. 1). The 15 µL digestion reaction was loaded in a 3% agarose gel. Following electrophoresis, fragments were visualized by staining with SIBR Green I (Sigma, Madrid, Spain). We even sequenced the 215 bp-PCR product of 10 patients to further validate the digestion assay and the concordance between the two methods was 100 %.

To make sure that our digestion reaction was performing well, we also amplified a 215 bp DNA fragment upstream the region of interest which happened to contain a restriction site for *Xcm* I. Forward and reverse primers (P3: 5'-GTATGTAACTACCCTGAGAGG-3'; P4: 5'-AGTGGCTTTTTGGAGGTGTCTC-3') were designed to generate a PCR product that also produced 170 bp- and 45 bp- long fragments after digestion. PCR conditions were similar to the ones mentioned above, except for the primer (20 pmoles) and the $MgCl_2$ (1.25 mM) concentrations.

Statistical analysis

Hardy-Weinberg equilibrium was checked by using the Tools for Population Genetic Analyses (TFPGA) program version 1.3. The Pearson chi squared test (or Fisher's exact test, where applicable) was performed to determine the significance of a frequency difference between groups with the SPSS software, version 10.0. A *p*-value lower than 0.05 was considered significant.

Results and discussion

Genotype frequencies were in Hardy-Weinberg equilibrium in cases and controls. Allele frequency for the T allele was slightly higher in the patients group: 0.074 versus 0.055. Eight out of the 54 SSc patients (14.8 %) were heterozygous for the SNP whereas the CT genotype was found in 6 out of the 55 controls (10.9%). Nevertheless, the difference did not reach statistical significance (p = 0.542, by Pearson chi-square test). None individual was found to be homozygous for the polymorphism neither in the patients nor in

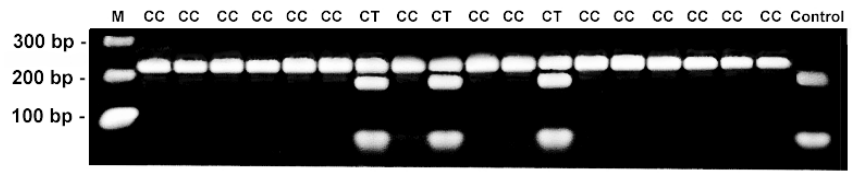

Fig. 1. Restriction enzyme digestion analysis of PCR products by *Xcm* I. Representative scleroderma individuals of each of the two genotypes found (CC or CT) are shown. Control: PCR fragment upstream the region of interest containing a restriction site for *Xcm* I. M: 100 bp molecular weight marker DNA. 3% agarose gel stained with SYBR Green I.

Table I. Distribution of those patients with certain immunological and clinical features according to their *PTPN22* genotype status.

	Immunological findings *		Clinical features *			
PTPN22 genotype	Anti-centromere antibodies	Anti-topoisomerase I antibodies	Pulmonary arterial hypertension	Interstitial lung disease	Scleroderma renal crisis	Cardiac involvement
CC	16 (94.1%)	11 (73,3%)	12 (100%)	14 (93.3%)	0 (0%)	15 (100%)
CT	1 (5.9%)	4 (26,7%)	0 (0%)	1 (6.7%)	1 (100%)	0 (0%)

* Percentages are calculated relative to the total number of patients who showed any of the immunological and clinical parameters considered.

the controls, which is somehow in accordance with the other studies reported so far in where the TT genotype has been occasionally detected. In fact, Orozco *et al.* (16) have recently found that the frequency of the TT genotype in an enlarged healthy Spanish population composed of 512 individuals was just of 0.8%, the heterozygous genotype being also similar to ours (12.3%). On the other hand, it is important to keep in mind that these percentages may differ from those observed in other populations with a different ethnic background. Thus, as emphasized by Begovich *et al.* (8), the T-allele frequency may be very low or even non-existent, as in the Han Chinese population (none of the 100 healthy people carried the SNP).

Differences were non-existent when comparison was made between those with limited scleroderma (5 out of 39 (12.8%) had the T-allele) and those with diffuse scleroderma (3 out of 13 (23.1%) were heterozygous for the polymorphism) ($p = 0.396$, by Fisher's exact test). It is also important to point out that the *PTPN22* polymorphism did not seem to be statistically associated with neither immunological nor clinical features (see Table I). Nevertheless, once antibody and clinical subsets were thus considered, study cohorts were in general too small to achieve a reliable negative conclusion.

Based on our data, we should conclude that this particular nucleotide change does not appear to be associated with SSc in the Spanish population. Other autoimmune diseases, such as multiple sclerosis, celiac disease, primary sclerosing cholangitis, primary Sjögren's syndrome, Crohn's disease, and giant cell arteritis, have not been associated to this SNP either (17-21). On the other hand, SSc (as all autoimmune disorders) is actually a complex disease and believing that only one intracellular enzyme could be responsible for protecting against autoimmunity seems to be quite simple. Since the contribution of genetics factors to the development of SSc is strongly supported, it is obvious that further research is needed in order for us to ascertain whether other genes are simultaneously involved in the pathology of SSc.

Given the fact that this SNP has, however, been linked to other autoimmune diseases, we could hypothesize that there may be a common mechanism underlying this association in all of them; such a mechanism would not then probably be shared with SSc. The expression of *PTPN22* is largely confined to haematopoietic tissues and all subtypes of normal human peripheral blood cells (PBMCs) (8). Nevertheless, it is unknown whether the missense mutation presented in this work affects the correct function of all these cells. Maybe some cells are more vulnerable to it than others. It would be interesting to study such an effect and see how it could be related to the different diseases based on the predominant affected cell subtypes found in each one.

References

1. LEROY EC, BLACK C, FLEISCHMAJER R *et al.*: Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; 15: 202-5.
2. TROJANOWSKA M: What did we learn by studying scleroderma fibroblasts? *Clin Exp Rheumatol* 2004; 22: S59-63.
3. POPE JE, SEIBOLD JR: International Conference on Systemic Sclerosis. *J Rheumatol* 1999; 26: 938-44.
4. ALBERT LJ, INMAN RD: Molecular mimicry and autoimmunity. *N Engl J Med* 1999; 341: 2068-74.
5. KRALING BM, MAUL GG, JIMENEZ SA: Mononuclear cellular infiltrates in clinically involved skin from patients with systemic sclerosis of recent onset predominantly consist of monocytes/macrophages. *Pathobiology* 1995; 63: 48-56.
6. FERRARINI M, STEEN V, MEDSGER TA, JR, WHITESIDE TL: Functional and phenotypic analysis of T lymphocytes cloned from the skin of patients with systemic sclerosis. *Clin Exp Immunol* 1990; 79: 346-52.
7. POSTLETHWAITE AE: Connective tissue metabolism including cytokines in scleroderma. *Curr Opin Rheumatol* 1995; 7: 535-40.
8. BEGOVICH AB, CARLTON VE, HONIGBERG LA *et al.*: A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am J Hum Genet* 2004; 75: 330-7.
9. HASEGAWA K, MARTIN F, HUANG G, TUMAS D, DIEHL L, CHAN AC: PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells. *Science* 2004; 303: 685-9.
10. BOTTINI N, MUSUMECI L, ALONSO A *et al.*: A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet* 2004; 36: 337-8.
11. KYOGOKU C, LANGEFELD CD, ORTMANN WA *et al.*: Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE. *Am J Hum Genet* 2004; 75: 504-7.
12. VELAGA MR, WILSON V, JENNINGS CE *et al.*: The codon 620 tryptophan allele of the lymphoid tyrosine phosphatase (LYP) gene is a major determinant of Graves' disease. *J Clin Endocrinol Metab* 2004; 89: 5862-5.
13. STEER S, LAD B, GRUMLEY JA, KINGSLEY GH, FISHER SA: Association of R602W in a protein tyrosine phosphatase gene with a high risk of rheumatoid arthritis in a British population: evidence for an early onset/disease severity effect. *Arthritis Rheum* 2005; 52: 358-60.
14. LEE AT, LI W, LIEW A *et al.*: The PTPN22 R620W polymorphism associates with RF positive rheumatoid arthritis in a dose-dependent manner but not with HLA-SE status. *Genes Immun* 2005; 6: 129-33.
15. SIMEON CP, ARMADANS L, FONOLLOSA V *et al.*: Mortality and prognostic factors in Spanish patients with systemic sclerosis. *Rheumatology* (*Oxford*) 2003; 42: 71-5.
16. OROZCO G, SANCHEZ E, GONZALEZ-GAY MA *et al.*: Association of a functional single-nucleotide polymorphism of PTPN22, encoding lymphoid protein phosphatase, with rheumatoid arthritis and systemic lupus erythematosus. *Arthritis Rheum* 2005; 52: 219-24.
17. BEGOVICH AB, CAILLIER SJ, ALEXANDER

HC *et al*.: The R620W polymorphism of the protein tyrosine phosphatase PTPN22 is not associated with multiple sclerosis. *Am J Hum Genet* 2005; 76: 184-7.

18. VIKEN MK, AMUNDSEN SS, KVIEN TK *et al*.: Association analysis of the 1858C>T polymorphism in the PTPN22 gene in juvenile idiopathic arthritis and other autoimmune diseases. *Genes Immun* 2005; 6: 271-3.
19. ITTAH M, GOTTENBERG JE, PROUST A *et al*.: No evidence for association between 1858 C/T single-nucleotide polymorphism of PTPN22 gene and primary Sjögren's syndrome. *Genes Immun* 2005; 6: 457-8.
20. VAN OENE M, WINTLE RF, LIU X *et al*.: Association of the lymphoid tyrosine phosphatase R620W variant with rheumatoid arthritis, but not Crohn's disease, in Canadian populations. *Arthritis Rheum* 2005; 52: 1993-8.
21. GONZALEZ-GAY MA, OLIVER J, OROZCO G, GARCIA-PORRUA C, LOPEZ-NEVOT MA, MARTIN J: Lack of Association of a Functional Single Nucleotide Polymorphism of PTPN22, Encoding Lymphoid Protein Phosphatase, with Susceptibility to Biopsy-Proven Giant Cell Arteritis. *J Rheumatol* 2005; 32: 1510-2.