



***TNFR1*-383 A>C polymorphism association with clinical manifestations in primary Sjögren's syndrome patients**

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ABSTRACT. Primary Sjögren's syndrome is an autoimmune disease affecting the function of exocrine glands. Tumor necrosis factor receptor-1 (TNFR1) is involved in apoptosis through extrinsic pathway initiation. The level of soluble TNFR1 is reported increased in rheumatoid arthritis, systemic lupus erythematosus, and primary Sjögren's syndrome patients. The *TNFR1* gene contains a polymorphism that replaced an adenine with a cytosine at the -383 in promoter region position. The *TNFR1*-383 A>C polymorphism has been associated with rheumatic diseases. We examined the association between the *TNFR1*-383 A>C polymorphism and TNFR1 soluble (sTNFR1) levels and

laboratory and clinical characteristics in primary Sjögren's syndrome patients. Eighty-two patients with primary Sjögren's syndrome classified using the American-European criteria and 84 healthy subjects were studied. Sjögren's Syndrome Disease Activity Index (SSDAI) and Sjögren's Syndrome Disease Damage Index were performed for all patients. Genotypic and allelic frequencies were similar in both groups ($P = 0.317$ and $P = 0.329$, respectively). sTNFR1 levels were similar in patients and healthy subjects ($P = 0.051$). High levels of C-reactive protein ($P = 0.045$) and rheumatoid factor ($P = 0.040$) in patients with the A>C genotype were observed. In these patients, the SSDAI score was higher than in A>A genotype carriers ($P = 0.045$). This is the first study that to examine the *TNFR1*-383 A>C polymorphism in primary Sjögren's syndrome patients. Clinical parameters and SSDAI index were associated in A>C genotype carriers. However, further studies with a larger sample are necessary to verify the association between primary Sjögren's syndrome and the *TNFR1*-383 A>C polymorphism.

Key words: Primary Sjögren's syndrome; *TNFR1*-383 A>C polymorphism; Laboratory and clinical characteristics; sTNFR1

INTRODUCTION

Primary Sjögren's syndrome (pSS) is an autoimmune disease influenced by various factors, including genetic background and the neurohormonal, viral, and immunologic systems. The pSS is related to epithelial damage and the loss of exocrine glandular functions (Ramos-Casals and Font, 2005; Anaya et al., 2006).

Apoptosis plays an important role in this disease by interacting with pro-inflammatory molecules such as tumor necrosis factor alpha (TNF α) (Hohmann et al., 1989; Lobito et al., 2006) and has a pleiotropic effect after binding to its transmembrane receptor (tumor necrosis factor receptor-1, TNFR1, or TNFR2) (Valle et al., 2010) that initiates apoptosis through the extrinsic pathway (Beg et al., 1996; Cope et al., 1997; Xanthoulea et al., 2004). Apoptosis is increased in chronic inflammatory diseases such as rheumatoid arthritis (RA), ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), and pSS (Bayley et al., 2003; Szodoray et al., 2004; Valle et al., 2010; Corona-Sanchez et al., 2012).

TNFR1 receptor is a type I transmembrane glycoprotein (Derré et al., 1991; Storey et al., 2002). Various studies have reported high sTNFR1 (soluble TNFR1) levels in the serum of AS, SLE, and AR patients. In pSS patients, TNFR1 has been reported elevated in the serum and in minor salivary gland biopsies (Szodoray et al., 2004).

TNFR1 is encoded by the *TNFR1* gene with its locus located at 12p13.2 (Derré et al., 1991; Xanthoulea et al., 2004). The *TNFR1* gene contains 10 exons and 9 introns (Derré et al., 1991). Various polymorphisms have been described in the promoter region that could modify the transcription rate. At position -383, a polymorphism replacing an adenine with a cytosine (*TNFR1*-383 A>C) has been associated with susceptibility to rheumatic diseases such as AS and RA. The relationship between clinical parameters, sTNFR1 levels, and the *TNFR1*-383 A>C polymorphism has not been investigated. The aim of this study was to examine

the association between the *TNFR1*-383A>C polymorphism and sTNFR1 levels and clinical parameters in pSS patients in a Mexican population.

MATERIAL AND METHODS

Patients and healthy subjects

Eighty-two patients with pSS were recruited at the Rheumatology Service of the Hospital General de Occidente in Zapopan, México. All patients fulfilled the 2002 American-European criteria for pSS (Vitali et al., 2002). In addition, the Sjögren Syndrome Disease Damage Index (SSDDI) and the Sjögren Syndrome Disease Activity Index (SSDAI) were applied to pSS patients (Vitali et al., 2007). As a control group, 84 healthy subjects (HS) who were matched for age and donors to the Blood Bank of the Hospital Civil "Fray Antonio Alcalde" were included. All participants were Mexican mestizos, which is defined as a mixed population composed of recent Amerindian, European, and African ancestors (Silva-Zolezzi et al., 2009).

Ethical considerations

Informed written consent was obtained from all pSS patients and HS before enrollment in the study according to the ethical guidelines of the 2012 Declaration of Helsinki (Macklin, 2012), and the Mexican Official Standard NOM-012-2012-SSA3 for research projects in humans (Secretaría de Gobernación, 2013) and the General Law in the field of health research of the United States of Mexico (Secretaría de Salud, 2013).

Laboratory test

Laboratory tests were conducted for the 2 groups (HS and pSS patients), including hematic biometry, blood chemistry, antinuclear antibodies, C-reactive protein (CRP), rheumatoid factor (RF), and sTNFR1. Additionally, anti-La and anti-Ro antibodies and Schirmer test results were collected from clinical records for the pSS group (Table 1).

Table 1. Demographic and clinical characteristics of pSS patients and HS.

Demographic and clinical characteristics	pSS (N = 82)	HS (N = 84)	P
Age in years (min-max)	58.1 (32-77)	53.1 (32-72)	-
Foci number (>1 foci/4 mm ²)	2.4 (1-8)	-	-
Schirmer test (mm in 5 minutes)	3.5 (0-5)	-	-
SSDAI (0-7)	1.5 (0-7)	-	-
SSDDI (0-4)	1.6 (0-4)	-	-
Anti-Ro (IU/mL)	15.39 (2.4-127.5)	-	-
Anti-La (IU/mL)	8.40 (1.2-11.37)	-	-
CRP (IU/mL)	3.5 (0.2-11.37)	2.8 (0.1-14.3)	NS
RF (IU/mL)	23.8 (2-242)	20.16 (20-21.8)	NS
ESR (mm/h)	25.1 (0-89)	25.6 (7-48)	NS
HB (g/dL)	13.6 (11.03-16.6)	14.1 (11-16.2)	0.013*
WBC (k/ μ L)	6 (0.6-10.5)	6.3 (3.01-11.3)	NS
PLT (k/ μ L)	274.9 (133-483)	278.4 (14.3-477)	NS

Anti-La: antibodies anti-La (Sjögren's syndrome A antigen), Anti-Ro: antibodies anti-Ro (Sjögren's syndrome B antigen), ESR: erythrocyte sedimentation rate, HB: hemoglobin, WBC: white blood cells, PLT: platelets, CRP: C reactive protein, RF: rheumatoid factor, SSDAI: Sjögren's Syndrome Disease Activity Index, SSDDI: Sjögren's Syndrome Disease Damage Index. Values are reported as averages (minimum maximum). *Significant, NS: not significant, P < 0.05 was considered to be significant.

PCR-RFLP screening of *TNFR1-383A>C* polymorphism

To identify the TNFR1 polymorphism, genomic (gDNA) was extracted from blood samples according to the technique of Miller with some modifications (Miller et al., 1988). Amplification was performed using the following primers: forward 5'-TTATTGCCCTTG-GTGTTTGGTTG-3' and reverse 5'-GAGGGGAAGAGTGAGGCAGTGTT-3' (Pitts et al., 1998; Wu et al., 2004; Valle et al., 2010; Corona-Sanchez et al., 2012). The polymerase chain reaction (PCR) reaction was performed in a 50- μ L final volume containing 1 μ L gDNA, 3 μ M each primer, 1.25 U/ μ L *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 μ L supplied 10X *Taq* reaction buffer using a 1X concentration for each PCR reaction (Invitrogen, Carlsbad, CA, USA), 1 mM MgCl₂, and 0.1 mM each dNTP (Invitrogen, Carlsbad, CA, USA). The PCR was performed by initial denaturation at 94°C for 3 min, followed by 35 amplification cycles at the following conditions: 94°C for 1 min for denaturalization, an annealing step at 62°C for 30 s, and an extension at 72°C for 1 min, and a final extension at 72°C in a Techne thermocycler (Model T-412; LabTech, Tampa, FL, USA). The final PCR product was a 199-bp fragment that was digested with 10 U *Bgl*II enzyme (Pitts et al., 1998; Wu et al., 2004; Valle et al., 2010; Corona-Sanchez et al., 2012) (New England BioLabs, Beverly, MA, USA) in the thermocycler for 4 h at 37°C. The wild-type genotype (A>A) corresponds to 135- and 64-bp fragments, the heterozygote genotype (A>C) is represented by the 199-, 135-, and 64-bp fragments, and the homozygote polymorphic genotype (C>C) corresponds to the 199-bp fragment. Restriction fragments were observed by electrophoresis on a silver nitrate-stained polyacrylamide gel (29:1 at 6%).

TNFR1 soluble levels quantification

Soluble TNFR1 (R&D Systems, Minneapolis, MN, USA) was measured using serum samples from pSS patients and HS (matched by age and sex) by enzyme-linked immunosorbent assay. The sTNFR1 range of detection was 7.8-500 pg/mL and the sensitivity of the assay was 1.2 pg/mL. sTNFR1 levels were calculated from a standard curve of sTNFR1.

Statistical analysis

Allele frequencies were determined using a counting method. Hardy-Weinberg equilibrium was tested using the Epi-Info v 7.0 software (Atlanta, GA, USA). The inter-group allele and genotype frequencies were analyzed using a contingency table. Non parametric comparisons between both groups were evaluated by the Mann-Whitney *U* test using SPSS v. 10.0 software (SPSS, Inc., Chicago, IL, USA). Graph-Pad Prism 6.0 software was used for the graphics. $P < 0.05$ was considered statistically significant, with a confidence index of 95%.

RESULTS

Demographic and clinical characteristics

Eighty-two pSS patients with a mean age of 58 years old were studied; all patients

were women. The average of the lachrymal flow was 3.5 mm in 5 min; average lymphocytic infiltration obtained from biopsies of the minor saliva gland was 2 foci in 4 mm². Damage and activity of the disease were evaluated (SSDDI and SDAI indexes). For SSDDI, oral, ocular and neurological damage were the most frequently observed (49, 34, 27%, respectively) and for SSDAI, constitutional and joint symptoms with 50 and 28%, respectively; changes in inflammation of the salivary glands was 12.5%. High anti-Ro levels were observed in the patients with the A>A genotype (Table 2).

Table 2. Laboratory and clinical characteristics of pSS patients stratified by genotype.

Laboratory and clinical data	pSS (N = 82)	Genotype A>A (N = 76)	Genotype A>C (N = 6)	P
Foci number (≥ 1 foci/4 mm ²)	2.40 (1-8)	3.06 (1-5)	2.01 (2-3)	NS
Schirmer test (mm in 5 minutes)	3.50 (0-5)	3.54 (0-5)	3.50 (2-5)	NS
SSDAI (0-7)	2.28 (1-8)	1.47 (1-5)	2.50 (0)	0.045*
SSDDI (0-4)	1.52 (0-5)	1.57 (0-5)	2.00 (1-3)	NS
Anti-Ro (IU/mL)	15.39 (2.4-127.5)	15.68 (2.4-127.5)	3.55 (2.4-127.5)	0.011*
Anti-La (IU/mL)	8.40 (1.2-11.37)	8.44 (1.2-11.37)	6.59 (1.2-11.37)	NS
CRP (IU/mL)	3.50 (0.2-11.37)	2.76 (0.2-11.4)	3.88 (1.29-7.59)	0.045*
RF (IU/mL)	24.30 (2-242)	21.82 (3-242)	44.00 (6-191)	0.040*

Anti-La: antibodies anti-La (Sjögren's syndrome A antigen), Anti-Ro: antibodies anti-Ro (Sjögren's syndrome B antigen), ESR: erythrocyte sedimentation rate, HB: hemoglobin, WBC: white blood cells, PLT: platelets, CRP: C reactive protein, RF: rheumatoid factor, SSDAI: Sjögren's Syndrome Disease Activity Index, SSDDI: Sjögren's Syndrome Disease Damage Index. Values are reported as averages (minimum maximum). *Significant, NS: not significant, P < 0.05 was considered to be significant.

Laboratory test

High levels of RF and CRP were found in the pSS patient. Anti-Ro antibody results were stratified by genotype and were 28, 7% for A>A and A>C, respectively and the anti-La antibodies were 27 and 9% for A>A and A>C, respectively. The distributions of genotypes and antibodies (anti-Ro and anti-La) were similar in A>A and A>C carriers.

Serum concentrations of TNFR1 in primary Sjögren's syndrome patients and healthy subjects

sTNFR1 levels were similar in both groups, with a mean of 6234.18 pg/mL for pSS patients and 5662.82 pg/mL in HS (P = 0.51, Figure 1A and B).

Analysis of the TNFR1-383 A>C polymorphism

The A>A genotype was the most frequently observed in pSS patients and HS (93 and 88%, respectively) and the A>C genotype frequency was 7 and 12%, respectively. Genotypic and allelic frequencies were similar in both groups (Table 3). The pSS patients with A>C genotype showed high levels of CRP and RF with P = 0.045 and P = 0.040, respectively (Table 2).

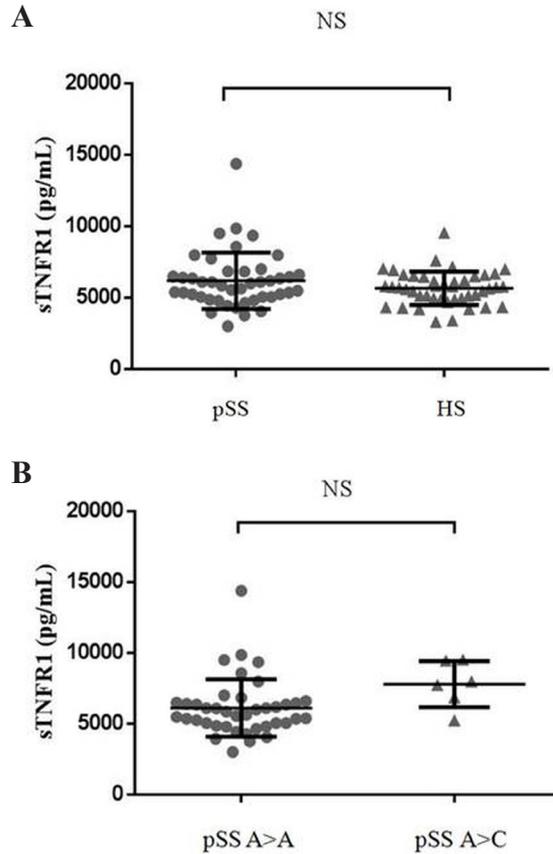


Figure 1. A. Soluble TNFR1 levels in pSS and HS. **B.** Soluble TNFR1 levels in patients stratified by genotype. pSS; primary Sjögren's syndrome, HS; healthy subjects. NS; no significant.

Table 3. Genotypic and allelic frequency of *TNFR1*-383 A>C in patients with pSS and HS.

	pSS, N = 82 [N (%)]	HS, N = 84 [N (%)]	χ^2
Genotypes			$\chi^2 (2) = 1.00$ P = 0.317
A>A	76 (93)	74 (88)	
A>C	6 (7)	10 (12)	
C>C	0 (0)	0 (0)	
Alleles			$\chi^2 (2) = 0.95$ P = 0.329
A	158 (91)	158 (91)	
C	6 (9)	10 (9)	

Genotypic and allelic frequencies were compared in pSS and HS. The C>C genotype was not found in the sample analyzed. P < 0.05 was considered significant.

DISCUSSION

TNFR1 is a molecule that can initiate the extrinsic apoptosis pathway and has been reported to be elevated in the peripheral blood of patients with rheumatic diseases such as AS and SLE (Derréet al., 1991; Szodoray et al., 2004; Valle et al., 2010; Corona-Sanchez et al., 2012).

RF and CRP levels were high in pSS patients; these results are similar with other previous reports in pSS patients (Derré et al., 1991; Szodoray et al., 2004; Ramos-Casals and Font, 2005; Valle et al., 2010; Corona-Sanchez et al., 2012). Anti-Ro and anti-La antibodies levels were similar to those reported by Hammi, et al. and Szododay, et al. and are related to the autoimmune process (Szodoray et al., 2004; Hammi et al., 2005).

The genotypic frequencies of *TNFR1* -383 A>C polymorphism were similar to those reported in RA and AS patients by Valle et al. (2010) and Corona-Sanchez et al. (2012); all of these patients were from western México. The A allele was most frequently observed in AR, AS, and SLE patients; these results suggest that the allele distribution is similar in different rheumatic diseases (Wu et al., 2004; Valle et al., 2010; Corona-Sanchez et al., 2012).

Patients with the A>C genotype showed the highest scores on the SSDAI and SSDDI indexes compared to patients with the A>A genotype, suggesting that patients carrying the C allele have more severe clinical presentations. However, SSDAI and SSDDI scores were low in pSS patients.

High sTNFR1 levels have been reported in RA patients from western Mexico (Valle et al., 2010). In 2004, Szodoray et al., reported high levels of soluble TNFR1 in HS in comparison with pSS; they studied only 9 patients with pSS and 9 HS matched by age and sex. Patients were selected based on American-European criteria, which was similar to our study, but they did not use the SSDAI and the SSDDI. Thus, since they did not examine activity or damage in pSS patients and because of the small number of patients, and we can not compare their data with our results.

In rheumatic diseases such as RA and SLE, high sTNFR1 levels have been reported (Wu et al., 2004; Valle et al., 2010), suggesting that TNFR1 plays an important role in the apoptosis pathway through pleiotropic and immunological activities (Valle et al., 2010). In pSS patients, we found that sTNFR1 levels were discretely elevated compared to HS, similar to another autoimmune diseases such as RA (Valle et al., 2010).

Anti-Ro and anti-La antibodies are markers considered in the American-European criteria such as diagnoses for pSS. In the present study, 31% of patients were positive for anti-Ro, which is similar to the results of Anaya and Skopouli, who reported the presence of anti-Ro in 40-80% of patients (Anaya et al., 1999; Skopouli et al., 2000).

In our study, 15% of patients were positive for anti-La antibodies, while Anaya and Skopouli reported a 30-60% positive rate for anti-La in pSS patients. This discrepancy may have resulted from the use of different methods used for detecting anti-Ro and anti-La antibodies. Anaya and Skopouli explain that the amplitude of ranges (30-60%) are because of heterogeneous laboratory detection methods used (Anaya et al., 1999; Skopouli et al., 2000).

In the present study, patients with the A>A genotype showed the highest levels of anti-Ro and anti-La antibodies compared with A>C genotype carriers. Nevertheless, in pSS, anti-Ro and anti-La antibodies are diagnostic markers and are not used for prognosis (Anaya et al., 1999; Skopouli et al., 2000). An important finding was that the A>C genotype showed a protective function in the development of anti-Ro and anti-La antibodies, which is similar to the results of Mullighan et al. (2004) in pSS in relation with *FAS-670* G>A in the polymorphic allele (A allele).

Finally, we did not identify an association between the *TNFR1* -383 A>C polymorphism and soluble TNFR1 levels. The levels of CRP and RF were high in A>C genotype carriers. Additionally, A>C genotype carriers showed higher scores than the A>A genotype on the

SSDAI index. The relationship between high levels of CRP and RF in association with the A>C genotype suggests relevant participation in the clinical presentation of pSS.

This is the first report to explore the association between the -383 A>C polymorphism and sTNFR1 levels and clinical parameters based on international scores of activity and damage with pSS; however, these possible associations should be investigated in further studies.

Future molecular genetics studies are necessary to explain the prognosis and clinical variability in pSS.

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