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Analysis of *IL10* haplotypes in primary Sjögren's syndrome patients from Western Mexico: Relationship with mRNA expression, IL-10 soluble levels, and autoantibodies

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ABSTRACT

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by lymphocytic infiltration of salivary and lacrimal glands. Interleukin-10 (IL-10) plays a role in autoimmune diseases by promoting B-cell activation and autoantibodies production. *IL10*-1082A > G, -819C > T, -592C > A polymorphisms and their haplotypes have been associated with IL-10 production. The aim of this study was to associate *IL10* haplotypes with mRNA expression and soluble IL-10 levels with susceptibility to pSS in 111 Mexican patients and 111 healthy subjects (HS). Primary Sjögren's syndrome patients showed high levels of sIL-10 ($p = 0.0001$ *vs* HS) correlating with anti-Ro and anti-La antibodies ($p < 0.05$). In addition, *IL10* mRNA expression in pSS was higher than HS (0.8 *vs* 0.1, $p = 0.1537$). However, no difference was observed in sIL-10 levels between haplotypes. Patients carriers of GCC haplotype showed higher mRNA expression than ACC + ATA (1.4 *vs* 0.6, $p = 0.2424$) and high foci number ($p = 0.04$ *vs* ACC). Our results suggest a strong relationship of *IL10* with pSS which is demonstrated by the increased mRNA expression and also high sIL-10 levels positively correlated with autoantibodies. Besides that, the GCC haplotype carriers expressed high mRNA. However, *IL10* haplotypes were not associated with sIL-10 in pSS from Western Mexico which suggest that diverse biological factors may regulate the *IL10* expression in pSS.
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1. Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by the lymphocytic infiltration to lachrymal and salivary glands conducing to an impaired secretory activity leading to the most important manifestations of the disease, keratoconjunctivitis sicca and xerostomia. B cell hyper reactivity is manifested by the presence of hypergammaglobulinemia and autoantibodies mainly against ribonucleoproteins (SSA/Ro and SSB/La) [1]. Several cytokines, such as interleukin-10 (IL-10), interleukin-6 (IL-6), and interferon gamma (IFN-γ) have been found increased levels in pSS patients suggesting an important role in the pathogenesis of the disease [2,3].

IL-10 is a pleiotropic and immunoregulatory cytokine. The human IL-10 is a homodimer of 37 kDa and each monomer consists of 160 amino acids [4]. The involvement of IL-10 in inflammatory processes and autoimmunity has been established [5–9]. Several polymorphisms in the *IL10* gene have been described, including

Abbreviations: ANA, antinuclear antibodies; bp, base pairs; CRP, C-reactive protein; DNA, deoxyribonucleic acid; dNTP's, deoxynucleotide triphosphate; ELISA, enzyme linked immunosorbent assay; ESR, erythrocyte sedimentation rate; ESSDAI, EULAR Sjögren's syndrome disease activity index; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gDNA, genomic deoxyribonucleic acid; HS, healthy subjects; HWE, Hardy–Weinberg equilibrium; IFN-γ, interferon gamma; IL-10, interleukin 10; IL-6, interleukin 6; kDa, kilo Dalton; LD, linkage disequilibrium; mRNA, messenger RNA; PCR, polymerase chain reaction; pSS, primary Sjögren's syndrome; RF, rheumatoid factor; RNA, ribonucleic acid; sIL-10, soluble IL-10; SSA/Ro, Sjögren's syndrome antibody A, also called Ro; SSB/La, Sjögren's syndrome antibody B, also called La; SSDAI, Sjögren's syndrome disease activity index; SSDDI, Sjögren's syndrome disease damage index.

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the single nucleotide polymorphisms (SNP) at positions -1082 A > G (rs1800896), -819 C > T (rs1800871), and -592 C > A (rs1800872) [10], which form three major haplotypes, GCC, ACC, and ATA. These haplotypes have been reported to be functionally important, since they are apparently associated with mRNA expression [11] as well as soluble IL-10 levels respect to no carriers [12,13].

In vitro stimulation of peripheral blood lymphocytes revealed that *IL10*-1082 GG genotype was associated with increased protein levels respect to AA genotype [14,15]. In a similar manner, the GCC haplotype showed higher transcriptional activity than the ATA haplotype in a luciferase reporter system and these results were in concordance with the levels of IL-10 in whole blood cultures from control subjects [13]. In addition, carriers of GCC haplotype have shown increased levels of *IL10* mRNA [11] as well as soluble IL-10 respect to no carriers [12].

Several studies have been conducted in order to investigate the role of *IL10* polymorphisms and haplotypes in the pathogenesis of autoimmune diseases [5–7,16–19]. In primary Sjögren's syndrome, the *IL10* polymorphisms and their haplotypes have been analyzed showing discordant results, -1082 GG and GCC haplotype have been associated as genetic risk factors for pSS in Caucasians [8,20,21], while ATA haplotype has been considered as risk factor in Asians [8,22]. However, some reports in Caucasians have not associated *IL10* polymorphisms and haplotypes with susceptibility to pSS [23–25].

The aim of this study was to analyze the association of *IL10* haplotypes with mRNA expression and soluble IL-10 levels with susceptibility to primary Sjögren's syndrome in patients from Western Mexico.

2. Materials and methods

2.1. Study subjects

The study included 111 patients with pSS, classified according to the American-European Consensus Group Criteria [26], from the Department of Rheumatology (HGO, SSJ, Zapopan, Jalisco, Mexico). The disease activity and damage was evaluated with Sjögren syndrome disease activity index (SSDAI) [27], Sjögren Syndrome Damage Index (SSDDI) [27], and EULAR Sjögren's syndrome disease activity index (ESSDAI) [28]. Information on treatment on the day of sampling was recorded. As a control group, 111 healthy subjects were included. All individuals were Mexican Mestizos defined as a person born in Mexico with Spanish-derived last name and Mexican ancestors traced back to the third generation including the own [29]. In both studied groups were determined a complete blood chemistry (Cell-Dyn 1700, Abbott Laboratories, Abbott Park, Illinois, USA), quantification of rheumatoid factor and C-reactive protein (CRP) by turbidimetry (BS120, Mindray, Shenzhen, China), and erythrocyte sedimentation rate (ESR) (performed by Wintrobe's method). All subjects signed voluntary participation in a written informed consent.

2.2. IL-10 serum levels and antibodies

Quantification of IL-10 soluble levels was performed using a high sensibility assay (ELISA kit R&D Systems, Minneapolis, MN, USA). The range of detection was 0.78–50 pg/mL, and the sensitivity of the assay was 0.17 pg/mL. Anti-Ro and anti-La autoantibodies were quantified using an ELISA kit (Orgentec Diagnostika GmbH, Mainz, Germany). The minimum detectable amount is 1.0 U/mL.

2.3. Analysis of IL10 promoter polymorphisms

The genomic DNA was isolated from peripheral blood, using the modified Miller's method [30]. The *IL10* polymorphisms at positions -1082 A > G (rs1800896), -819 C > T (rs1800871), and -592 C > A (rs1800872) were genotyped by polymerase chain reaction and restriction fragment length polymorphism using primers previously reported [31–33].

The PCR reaction was performed in a final volume of 25 μL containing 1 μg of gDNA for -1082 A > G and -819 C > T, and 0.5 μg for -592 C > A, 3 mM of each oligonucleotide, 0.5 U/μL of *Taq* DNA polymerase, 1× of supplied buffer enzyme, 2 mM of $MgCl_2$ (Invitrogen Life Technologies, Carlsbad, CA, USA), and 2.5 mM of each deoxynucleotide triphosphate (dNTP's) (Promega Corporation, Madison, WI, USA). The following cycling conditions were used: Initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s for denaturation, 60 °C for 30 s for annealing (56 °C for *IL10*-592 C > A), extension at 72 °C for 30 s and, finally, 72 °C for 1 min was used for an ending extension.

The PCR amplified fragments, 102 base pairs (bp) for -1082 A > G [31], 348 bp for -819 C > T [32], and 588 bp for -592 C > A [33], were digested with *EcoNI*, *MslI*, and *RsaI*, respectively, according to the manufacturer instructions (New England Biolabs, Beverly, MA, USA). The restriction fragments were observed on a 6% polyacrylamide gel (7% for *IL10*-592 C > A) stained with silver nitrate. For -1082 A > G polymorphism, the restriction patterns generated were 102 bp for AA; 102, 82, 20 bp for AG; and 82, 20 bp for GG genotype. For -819 C > T polymorphism, the digested fragments were 288 and 60 bp for CC; 348, 288, and 60 bp for CT; and 348 bp for TT. Respect to -592 C > A polymorphism, the genotypes were identified for 305, 233, 42, and 8 bp for CC; 305, 240, 233, 65, 42, and 8 bp for CA; 240, 233, 65, 42, and 8 bp for AA.

2.4. Haplotype inference

Haplotypes and their frequencies were inferred using EMHAPFRE software. Block structure was examined using the Haploview software version 4.2 (Broad Institute, Cambridge, MA, USA). The D' values and r^2 values for all pairs of SNPs were calculated, and the haplotype blocks were estimated using Haploview software version 4.2 which is based on the Lewontin corrected coefficient [34,35].

2.5. Quantitative mRNA expression

RNA was isolated from peripheral blood of pSS and HS, according to Chomczynski and Sacchi technique [36]. For mRNA analysis, an aliquot containing 1 μg of total RNA was transcribed reversely using oligo-dT and M-MLV reverse transcriptase following the manufacturer instructions (Promega, Madison, WI, USA). The quantification of *IL10* mRNA was by real time PCR using *Taqman* hydrolysis probes (Roche Applied Science, Penzberg, Germany). The primers and probes for quantification were obtained by a design program of Roche Applied Science (*Universal ProbeLibrary Assay Design Center*), using the sequence of *IL10* mRNA obtained from NCBI ID number: NM_000572.2 (67 number test, Cat. No. 04688660001). The *GAPDH* was used as reference gene (Cat. No. 05190541001). The PCR reaction was performed on a Light Cycler Nano System (Roche Applied Science, Penzberg, Germany). The $2^{-\Delta Ct}$ ($\Delta Ct^{IL10} - \Delta Ct^{GAPDH}$) method was used to analyze *IL10* mRNA expression [37].

2.6. Statistical analysis

Statistical significance was considered at the level of $p \leqslant 0.05$. Hardy–Weinberg equilibrium (HWE) was examined by χ^2 test.

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