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Short Communication

The extrapituitary prolactin promoter polymorphism is associated with rheumatoid arthritis and anti-CCP antibodies in Mexican population

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ABSTRACT

Prolactin (PRL) is a hormone–cytokine that has been involved in autoimmunity due to its immunoregulatory and lymphoproliferative effects. It is produced by various extrapituitary sites including immune cells, under control of a superdistal promoter that contains a single nucleotide polymorphism −1149 G/T previously associated with rheumatoid arthritis (RA) susceptibility in European population. The aim of this study was to investigate the association of the extrapituitary *PRL* −1149 G/T promoter polymorphism with clinical parameters, clinical activity and disability indices in RA patients from Western Mexico and to analyze the *PRL* mRNA expression according to the *PRL* −1149 G/T promoter polymorphism in total leucocytes from RA patients and controls. We conducted a case–control study that included 258 RA patients and 333 control subjects (CS). The DNA samples were genotyped using the PCR–RFLP method and the *PRL* mRNA expression was determined by quantitative real time PCR. PRL serum levels and antibodies to cyclic citrullinated peptides (anti-CCP) were measured with ELISA. We found significant differences in the genotype ($p = 0.022$) and allelic ($p = 0.046$) distribution of the polymorphism between RA patients and control subjects. According to the dominant genetic model, there is an association between the T allele (GT + TT genotypes) and decreased RA susceptibility in comparison to the G allele carriers (GG genotype) (OR 0.64, 95% CI 0.45–0.92; $p = 0.011$). The T allele carriers (GT + TT genotypes) had lower titers of anti-CCP antibodies in comparison to the G allele carriers (GG genotype) (median, 66 U/mL *vs.* 125 U/mL; $p = 0.03$). Furthermore, the GG homozygotes had higher *PRL* mRNA expression in comparison to the GT heterozygotes, and this latter with respect to the TT homozygotes, in both groups (RA: 1 > 0.72 > 0.19; CS: 1 > 0.54 > 0.28). However, PRL serum levels were similar in both groups. Our results suggest that the *PRL* −1149 T allele is a genetic marker for decreased RA susceptibility and is associated with lower titers of anti-CCP antibodies in Mexican population. We also suggest influence of genotype upon *PRL* mRNA expression.

1. Introduction

Rheumatoid arthritis (RA) is the most common systemic autoimmune disease affecting 0.5% to 1% of the adult population worldwide (Silman and Pearson, 2002). It is characterized by chronic inflammation of the synovium, which leads to progressive joint destruction and functional disability (Firestein, 2003). Its etiology is unknown, but it has been proposed that genetic, hormonal and environmental factors contribute on its development (McInnes and Schett, 2011).

The higher incidence of RA in women (3:1 ratio) has generated much interest about the hormonal influence on disease risk (Oliver and Silman, 2009). Prolactin (PRL) is an inflammatory hormone with cytokine properties postulated to play a role in the pathogenesis of

Abbreviations: PRL, prolactin; RA, rheumatoid arthritis; anti-CCP, anti-cyclic citrullinated peptide; SNP, single nucleotide polymorphism; CS, control subjects; RF, rheumatoid factor; hsCRP, high sensibility C reactive protein; ESR, erythrocyte sedimentation rate; HPRL, hyperprolactinaemia; DAS-28, disease activity score 28; spanish HAQ-DI, spanish version of health assessment questionnaire disability index; NSAIDs, non steroidal anti-inflammatory drugs; DMARDs, disease modifying anti-rheumatic drugs.

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RA; several studies have reported increased PRL serum concentrations in patients with RA (Ram et al., 2004; Seriolo et al., 2002) and it is suggested that PRL contributes in the increased risk of postpartum flares and disease development, since PRL levels augment during breastfeeding (Barrett et al., 2000). It is produced in the pituitary gland and also in extrapituitary sites, including tissues and cells of the immune system upon of which exerts immunostimulatory and lymphoproliferative effects. PRL acts synergistically with IL-2 promoting the proliferation of T, B and NK cells (Clevenger et al., 1990; Matera et al., 1992), stimulates the production of IFN-γ in T cells (Matera and Mori, 2000), promotes dendritic cell maturation (Carreno et al., 2004), enhances the synthesis of antibodies and regulates the mechanisms of tolerance in B cells (Peeva et al., 2004; Saha et al., 2011), among other effects. Furthermore, its local production by lymphocytes infiltrating the synovium in RA, and its ability to stimulate fibroblast-like synovial cells to synthesize proinflammatory cytokines and cartilage degrading enzymes (matrix metalloproteinases), thereby promoting the pathogenesis of the disease have been confirmed (Nagafuchi et al., 1999).

The *PRL* gene has two independent promoter regions: a proximal that regulates the transcription in the pituitary, and a superdistal that regulates the transcription in extrapituitary sites (Ben-Jonathan et al., 2008). This extrapituitary promoter contains a single nucleotide polymorphism (SNP) at position −1149 G/T (rs1341239) proposed to affect *PRL* transcription in lymphocytes (Stevens et al., 2001). This polymorphism has been associated with susceptibility to autoimmunity, particularly with systemic lupus erythematosus (SLE) (Stevens et al., 2001) and RA in European populations (Fojtíková et al., 2007; Lee et al., 2009).

To date, there are no studies evaluating the association of the extrapituitary *PRL* promoter polymorphism with RA in Mexican patients. Since this population is considered Mestizo, the result of a mixture of European, Amerindian and African genes (Rangel-Villalobos et al., 2008), conducting an examination of this population is additionally a way to investigate several genetic influences simultaneously and to determine whether the *PRL* mutations persist outside of the previously tested European populations.

Based on this, we decided to investigate the association of the extrapituitary *PRL* −1149 G/T promoter polymorphism with clinical parameters, clinical activity and disability indices in RA patients from Western Mexico and to analyze the *PRL* mRNA expression according to the *PRL* −1149 G/T promoter polymorphism in total leucocytes.

2. Materials and methods

2.1. RA patients and control subjects

A case–control study was conducted in the Functional Immunogenetics Research Group, Universidad de Guadalajara, Jalisco, Mexico. The RA patients group included 258 subjects from Western Mexico fulfilling the 1987 American College of Rheumatology criteria for RA (Arnett et al., 1988), enrolled from the rheumatology service in the Hospital General de Occidente, Zapopan, Jalisco, Mexico and the Hospital Civil de Guadalajara "Fray Antonio Alcalde", Guadalajara, Jalisco, Mexico. Upon inclusion, all patients were examined by a physician according to a structured protocol that consisted of physical assessment, medical record and evaluation of 28 joints to determine the clinical activity according to the Disease Activity Score-28 (DAS-28) (Prevoo et al., 1995); the extent of disability was estimated according to the Spanish version of Health Assessment Questionnaire-Disability Index (Spanish HAQ-DI) (Cardiel et al., 1993). The control subjects (CS) group comprised 333 unrelated, healthy individuals identified by self-report recruited from Western Mexico general population. The study was conducted according to the ethical guidelines of the Declaration of Helsinki, and all subjects in the study gave informed written consent.

2.2. Laboratory assessment

A sample of peripheral blood was drawn from RA patients and CS in order to determine clinical parameters. Rheumatoid factor (RF) and high sensibility C reactive protein (hsCRP) were quantified by a turbidimetric assay (COD31030 and COD31029, respectively; BioSystems, Spain); the erythrocyte sedimentation rate (ESR) was measured using the Wintrobe method. Individuals with serum values >20 IU/mL were considered as RF positive (cut-off defined by the manufacturer, BioSystems, Spain).

2.3. Anti-CCP quantification

Anti-cyclic citrullinated peptide (anti-CCP) antibodies were measured in serum samples from all patients and controls with a second-generation enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (FCCP400; DIASTAT, Axis-Shield Diagnostics, UK). The anti-CCP levels were calculated from a standard curve (0–200 U/mL) and the samples with values above the calibration curve were rerun after dilution. Individuals with serum values >5 U/mL were regarded as anti-CCP positive (cut-off defined by the manufacturer, Axis-Shield Diagnostics, UK).

2.4. PRL quantification

In order to obtain reliable results, a stricter selection of individuals was done for the measurement of PRL serum levels; 106 female RA patients and 106 female CS matched by age were analyzed. Exclusion criteria included pregnancy, endocrine, renal or any other disease and medication known to affect serum PRL concentrations. PRL was measured with ELISA according to the manufacturer's instructions (EIA-1291; DRG, International). Concentrations were calculated from a standard curve (0–200 ng/mL) and none of the samples reached values above the highest standard. Hyperprolactinaemia (HPRL) was defined as serum concentration >20 ng/mL.

2.5. PRL −1149 G/T polymorphism genotyping

Genomic DNA (gDNA) was extracted from leukocytes obtained from a sample of 3 mL of peripheral blood according to the Miller modified method (Miller et al., 1988). The *PRL* −1149 G/T polymorphism was detected using the PCR–RFLP method. Amplification of the polymorphic promoter fragment was done with the following primers: 5′-AGA ATT GGA GTT CCA GTG CC-3′ (forward) and 5′-ATC ACA CTC AAC CAG TTG GC-3′ (reverse) (Donn et al., 2002). PCR was carried out in a final volume of 25 μL containing 100 ng of gDNA, 0.04 μM of each primer, 0.6 U *Taq* polymerase, 1× enzyme supplied buffer, 4 mM $MgCl_2$, and 0.1 mM of dNTPs (Invitrogen™ life technologies). The cycling parameters were as follows: initial denaturalization at 95 °C for 5 m, 35 cycles including denaturation at 95 °C, annealing at 58 °C, extension at 72 °C for 30 s each and final extension at 72 °C for 5 m. The resulting PCR product (338 bp size) was visualized on a 6% polyacrylamide gel stained with 2% $AgNO_3$. 5 μL of the amplified fragment was incubated with 3 U of *ApoI* enzyme (New England, BioLabs, Inc.) at 50 °C for 1 h and the restriction fragments were visualized after electrophoresis in a 6% polyacrylamide gel and staining with 2% $AgNO_3$. The GG homozygote was identified as 2 fragments (280 and 58 bp), the GT heterozygote as 3 fragments (338, 280, 58 bp) and the TT homozygote as a single fragment of 338 bp. As quality control, samples of each genotype were randomly selected and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed in an automated sequencer (ABI Prism 310, Applied Biosystems, California).