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Tumor necrosis factor–α is a common genetic risk factor for asthma, juvenile rheumatoid arthritis, and systemic lupus erythematosus in a Mexican pediatric population

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ABSTRACT

There is a great deal of evidence that points to the association of the tumor necrosis factor-alpha (*TNF-α*) gene as a common genetic factor in the pathogenesis of diseases that are caused by inflammatory and/or autoimmune etiologies. Two single nucleotide polymorphisms (SNPs) identified in the *TNF-α* promoter region have been associated with disease susceptibility and severity. We investigated whether −308G/A and −238G/A *TNF-α* polymorphisms were associated with asthma, systemic lupus erythematosus (SLE), and juvenile rheumatoid arthritis (JRA) in a pediatric Mexican population. In a case-control study of 725 patients (asthma: 226, JRA: 171, and SLE: 328) and 400 control subjects, the participants were analyzed using the allelic discrimination technique. The genotype distribution of both *TNF-α* polymorphisms was in Hardy-Weinberg equilibrium in each group. However, there were significant differences in the allele frequency of *TNF-α*-308A between the patients and the healthy controls. This allele was detected in 2.9% of the controls, 6.0% of asthmatic and JRA patients ($p = 0.002$ and $p = 0.0086$), and 6.7% of SLE patients ($p = 0.00049$); statistical significance was maintained after ancestry stratification (asthma: $p = 0.0143$, JRA: $p = 0.0083$, and SLE: $p = 0.0026$). Stratification by gender showed that the risk for the −308A allele in asthma and JRA was greater in females (OR = 4.16, $p = 0.0008$ and OR = 4.4, $p = 0.0002$, respectively). The *TNF-α* -238A allele showed an association only with JRA in males (OR = 2.89, $p = 0.004$). These results support the concept that the *TNF-α* gene is a genetic risk factor for asthma, SLE, and JRA in the pediatric Mexican population.

1. Introduction

There is strong evidence supporting the role of cytokine and cytokine receptor genes in the pathogenesis of inflammatory and/or autoimmune diseases [1–3]. The differences in the production of these cytokines, as well as the participation of other genetic factors, likely account for the marked clinical heterogeneity among such diseases. One of the major cytokines that has been intensely investigated is the tumor necrosis factor–α (TNF-α), a multifunctional cytokine T helper 1 (Th1) molecule [4,5]. TNF-α plays a central role in inflammation; it induces the expression of other proinflammatory molecules, chemotactic cytokines and adhesion factors [5,6]. *In vivo* and *in vitro* studies have shown that high levels of TNF-α lead to exacerbation of the inflammatory response. This, together with its potent immunomodulator activities, has been suggested as important to the pathogenesis of diseases such as asthma, systemic lupus erythematosus (SLE) and rheumatoid ar-

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thritis (RA) [7–9]. Several studies have shown that high serum TNF-α levels are linked to several pathologic features such as hyperresponsiveness in asthma, joint destruction in RA and the production of autoantibodies in SLE [10–16]. Furthermore, TNF-α exerts a wide variety of immunoregulatory effects on several cell types such as B and T lymphocytes, dendritic cells, and mast cells, which are relevant to the pathogenesis of these diseases [6,9,11,14].

The *TNF-α* gene is a member of the TNF superfamily located within the class III region of the human major histocompatibility complex (MHC) on chromosome 6p21 [17]. Several SNPs have been identified in the *TNF-α* gene [18–20]. However, the most extensively studied are the biallelic *TNF-α* 308 G/A and *TNF-α* 238 G/A polymorphisms in the promoter region [21–40]. There is no strong evidence to date showing that the −308 site is a binding site for transcription factors, or is correlated with protein expression [41,42]. However, the *TNF-α*-308A allele appears to be associated with a several fold increase in transcriptional activity and has been associated with increased levels of TNF [13,43,44]. In fact, recent studies have shown that individuals carrying the GA genotype have a greater amount of *TNF-α* mRNA, and serum level concentrations, than individuals with the GG genotype; the AA genotype was not present in their sample [10,45,46]. Similarly, Louis *et al.* [47], reported an *in vitro* study where cells stimulated with lipopolysaccharide, from individuals with the *TNF-α*-308A allele, expressed more TNF-α than did the cells from individuals that were homozygous for the G allele. On the other hand, although the effect of other polymorphisms on gene regulation has not been clearly defined, current data suggest that the *TNF-α*-238 polymorphism appears to influence gene function; for example, the *TNF-α*-238G allele has been shown to create a CpG methylation site that could modulates the gene transcriptional rate [48].

Many independent studies, and several meta-analyses, have shown that these polymorphisms are associated with the susceptibility and severity of asthma, SLE and RA, particularly the *TNF-α*-308A allele [49–51]. However, these observations have not been replicated in all populations, suggesting the possibility of ethnic differences [21,22,27,30,33,52–55].

Given the known importance of *TNF-α* in inflammatory and/or immune functions and the variation in susceptibility to immune disorders in different ethnic groups, we investigated the association of this gene with the development of asthma, juvenile rheumatoid arthritis (JRA) and SLE in a pediatric Mexican population.

2. Subjects and methods

2.1. Study population

A total of 226 patients with asthma, 171 with JRA and 328 with SLE, were enrolled into this study. All patients were less than 16 years of age and were recruited from four tertiary level institutions located in Mexico City. The diagnosis of asthma was made based on the definition used by the American Thoracic Society and the Global Initiative for Asthma (GINA) criteria. The patients with JRA and SLE fulfilled the American College of Rheumatology (ACR) criteria for the diagnosis. Because there is no evidence of a correlation between TNF alleles and age [56], we included 400 unrelated healthy subjects more than 18 years of age, without autoimmune or inflammatory disease as healthy controls. The study was conducted with the approval of the ethics and research committees from all four participating institutions. Informed parental consent was obtained for all patients. Parents provided consent for the children's participation and the children assented.

2.2. Genotyping analysis

Genomic DNA was extracted from blood samples using the standard salting out technique (QIAgen Systems, Inc., Valencia, CA, USA). Genotyping analyses of *TNF-α*-308 G/A and *TNF-α*-238 G/A polymorphisms were performed using the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA). The polymerase chain reaction (PCR) was carried out using the ABI PRISM 7900 system. The primers were added in a PCR mix consisting of 10 ng of genomic DNA, 2.5 μl of TaqMan master mix, 0.125 μl of 20X assay mix, and ddH2O up to a final volume of 5 μl. The amplification protocol included denaturing at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute. The genotype of each sample was assigned automatically by measuring the allele-specific fluorescence using SDS 2.2.3 software for allelic discrimination (Applied Biosystems, Foster City, CA).

The overall genotype call rate by TaqMan was 99.9%; only one sample for asthma and SLE diseases were not successful in one of the SNPs analyzed. A total of 426 randomized samples were genotyped in duplicate for both polymorphisms; the genotyping reproducibility was 100%. To confirm the *TNF-α*-308 genotype, 100 random samples, and the only AA homozygote case, were sequenced.

The PCR products were sequenced directly with a DNA Sequencing Kit with the Big Dye Terminator on an automated ABI PRISM 3100 DNA sequencer (Applied Biosystems). The forward and reverse primer sequences were 5′-AAACAGACCACAGACCTGGTC-3′ and 5′-GGGAGGATGGGAGTGTGAG-3′ [29]. We also evaluated a panel of 10 informative ancestry markers (rs4884, rs2695, rs17203, rs2862, rs3340, rs722098, rs203096, rs223830, rs1800498, and rs281478) that mainly distinguish Amerindian and European ancestry ($\delta > 0.44$). These markers have been validated in prior case-control studies in the Mexican population [57,58].

2.3. Statistical analysis

The FINNETI program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to test Hardy-Weinberg equilibrium for genotype distributions in the cases and controls. Odds ratios (OR) with 95% confidence intervals (95% CIs) were calculated using allele frequencies for the case patients and controls. The Stat-Calc program (Epic Info 2005 V.3.2; Centers of Disease Control and Prevention, Atlanta, GA) was used for all statistical calculations. The χ^2 value was calculated from 2×2 contingency tables, and values of $p < 0.05$ were considered statistically significant. The ADMIXMAP program [59] was used to test the possible effect of population stratification, as previously described in prior studies [60,61] and performed with the two primary parental populations of Mexican-Mestizo (Amerindian and European). Because of the almost complete absence of AA homozygotes in both SNPs, we used a dominant model of inheritance. We included a gender covariate as a potential confounder and the genotype was considered a fixed factor, using the logistic regression fits in the ADMIXMAP. However, age was not considered a confounding factor, since there is no evidence that the *TNF-α* polymorphisms may be related to a lesser life span.

The statistical power for asthma, JRA, and SLE studies was estimated using QUANTO software (version 1.2; http://hydra.usc.edu/GxE/) and all of them reached more than 80% under dominant model.

3. Results

The asthmatic patients ranged in age from 5 to 17 years and fulfilled the GINA criteria for asthma. The SLE and JRA cohorts included patients from less than 16 years of age at onset of disease and fulfilled the ACR criteria. The mean (±SD) age at onset of asthma, JRA, and SLE were 8.4 ± 2.8, 8.7 ± 2.46, and 11.62 ± 2.46 years, respectively. The female/male gender distribution was 83 (37%)/143 (63%) among asthmatic patients, 97 (57%)/74 (43%) in the JRA patients, and 274 (84%)/54 (13%) in the SLE patients. The gender ratio for the 400 healthy, ethnically matched controls was 1:1 (female:male); all were more than 18 years of age. Because