No Association of Eight TNFAIP3 Single Nucleotide Variants to Rheumatoid Arthritis in Native Mexicans

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Short Report

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No association of eight *TNFAIP3* single nucleotide variants to rheumatoid arthritis in Mexicans

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease of autoimmune origin associated with many genetic traits, including genes related to the control of inflammation. The A20 protein, encoded by the *TNFAIP3* gene, is a negative regulator of NF-κB-mediated inflammation. Several single nucleotide variants (SNVs) of *TNFAIP3* are associated with susceptibility to RA in different ethnic groups, but not in Chileans, the only Latin Americans studied thus far. **Objective.** To examine the association of eight *TNFAIP3* SNVs, four of them not previously studied, with RA in Mexican patients. **Materials**. We studied 471 RA patients (ACR-EULAR 2010) and eight *TNFAIP3* SNVs: including, rs10499194C/T, rs6920220G/A and rs2230926T/G, which are associated with RA in European and Asian patients, in addition to rs373421182G/C, rs139054966T/G, rs5029924C/T, rs59693083A/G and rs61593413T/A, not previously examined in RA. All SNVs were evaluated by means of an allelic discrimination assay and TaqMan probes. **Results.** The allelic and genotypic frequencies of all SNVs examined were similar between cases and controls, and none of them was associated with RA under the allelic, codominant, dominant, and recessive models was observed. **Conclusion.** Our data indicate that the *TNFAIP3* SNVs evaluated herein are not risk factors for RA in Mexican subjects.

Keywords: single nucleotide variants, rheumatoid arthritis, susceptibility.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease (AD) characterized by chronic and symmetric joint inflammation leading to progressive cartilage degradation and functional disability. Cells involved in the pathogenesis of RA include $CD4^+$ T cells, macrophages, and neutrophils, among others, that, together, contribute to the inflammatory process [1]. These cells release inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-17, tumor necrosis factor (TNF), and others [2-4]. Of these, TNF is a major mediator of the immunopathology in RA, mostly through activation of the NF-κB transcription factor [1, 5, 6]. NF-κB activity is regulated at different levels, with the A20 protein (encoded by *TNFAIP3*) as a major negative regulator of its activity [2, 7, 8] though different mechanisms, including removal of K63-linked ubiquitin chains from the receptor interacting protein 1 (RIP1), an adaptor protein involved in the TNF receptor 1 (TNFR1) signaling pathway. Moreover, A20-dependent conjugation of RIP1 to K48-linked polyubiquitin chains leads to its proteasomal degradation, preventing phosphorylation of IKKβ and, therefore, no activation of the Iκ-B kinase, avoiding nuclear translocation of NF-κB. This prevents the expression of genes involved in inflammation [9]. On the other hand, mice deficient in A20 die prematurely from multi-organ inflammation because of increased NF-κB signaling in *in vitro* studies. Moreover, A20-deficient mouse embryonic fibroblasts exhibit persistent NF-κB signaling as evidenced by increased IκBα degradation [10].

Genome-wide association (GWAS) and candidate gene studies, respectively, have identified and replicated associations of different single nucleotide variants (SNVs) of *TNFAIP3* with susceptibility to several AD, including RA [11-15]. Although the functional

consequences of some variants on A20 functions are unknown, others, such as rs2230926T/G (Phe127Cys) leads to increased NF-κB activity and an enhanced response to pro-inflammatory cytokines and TNF-induced gene transcription [3, 16].

At least three *TNFAIP3* SNVs have been widely studied in RA patients, primarily in Caucasians and Asians-derived populations. Two of them, rs2230926T/G and rs6920220G/A, have been associated with an increased risk for RA in Asians and Caucasians, respectively, whereas *TNFAIP3* rs10499194C/T is associated with protection against RA in Caucasians [15]. *TNFAIP3* rs6920220G/A and rs10499194C/T have a low or no effect in Asians [14, 15]. Although studies on the functional consequences of these variants are scarce, it has been found that the non-synonymous G allele (Cys) of rs2230926T/G, located in exon 3 of *TNFAIP3*, leads to a modest but consistently lower inhibition of TNF-dependent NF-κB activity compared to the T allele (Phe).

Association studies of different genes to RA in Latin-Americans are scarce [17]. Mexicans constitute a heterogeneous population with varying genetic contributions from Amerindians, European-Caucasians, and Africans [18, 19]. Here we examined the association of eight *TNFAIP3* SNVs (rs10499194C/T, rs6920220G/A, rs2230926T/G, rs373421182G/C, rs139054966T/G, rs5029924C/T, rs59693083A/G, and rs61593413T/A) in patients with RA from central Mexico. These SNVs, except for rs2230926T/G, were chosen because of their proximity to potential transcription factor-binding sites in the *TNFAIP3* promoter. In addition, some of these SNVs have been associated with other inflammatory diseases, but their role in RA is unknown [20].

Material and Methods

Study population

Our study group consisted of 471 patients with RA recruited at the Rheumatology Service of Hospital Juárez de México, who fulfilled the 2010 ACR-EULAR criteria [21], as well as 499 healthy subjects with no chronic degenerative or infectious diseases and no history of ADs were included as controls. All patients and controls were females, all of them were unrelated and over 18 years old who gave their informed consent to accept to participate in the study. The study was performed in accordance with the guidelines of the Declaration of Helsinki and was approved by the Research and Bioethics Committees of Hospital Juárez de México (HJM-0446/18-I).

Isolation of genomic DNA

From each of the cases and controls, 8 mL peripheral blood were collected in tubes with EDTA. Subsequently, genomic DNA was isolated by the commercial kit Invisorb Blood Universal Kit (Stratec molecular GmbH, Berlin, Germany) in which leukocytes were lysed and proteins were removed by digestion, then the DNA was precipitated, washed, and resuspended in nuclease-free water. DNA samples were quantified, diluted, and stored at -20 °C until use.

Genotyping

Eight *TNFAIP3* variants were genotyped: rs2230926T/G, rs104999194C/T, rs6920220G/A rs59693083A/G, rs61593413T/A, rs139054966T/G, rs5029924C/T, and rs373421182G/C. Figure 1 shows the distribution of the SNVs evaluated in this study along the *TNFAIP3* gene. Allelic discrimination was achieved by means of TaqMan probes. Each assay included 10 ng of genomic DNA, 2.5 µl of 2x master mix, 2.437 µl of DNase-free water, and 0.0625 µl of probe in a final volume of 5µl.

Real time PCR was implemented in the CFX96 C1000 equipment from BioRad (Hercules, CA, USA) and the Quant Studio 12K Flex Real-Time (Thermo Fisher Scientific, Foster C. CA, USA). The run conditions were: 1 cycle PCR 60°C/2 minutes, 95 °C denaturation for 10 minutes, with 40 cycles of denaturation for 15 seconds and alignment and extension for 1 minute at 60 °C. Allelic discrimination was carried out with the Bio-Rad CFX Manager 3.1 software (Applied Biosystems, CA, USA).

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) for each *TNFAIP3* SNVs in RA and controls was calculated by using the Finetti software (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl), a *p*-value <0.05 in controls was indicative of a deviation from HWE. The Chi-square test was used to estimate the OR value. The Epidat 3.1 software was used to evaluate the allelic and codominant models. The Haploview (V. 4.2) software was used to determine linkage disequilibrium (LD) and to evaluate haplotypes. The statistical power in our case-control study was calculated with Quanto program (http://biostats.usc.edu/cgi-bin/DownloadQuanto.pl).

Results

Demographic features

A total of 471 RA patients and 499 healthy control women were included in the present study. The mean ages of RA patients and controls were similar (51±14, RA vs. 52.7±7.9, controls) and all of them were from Central Mexico including Mexico City, State of Mexico, Morelos, and Puebla.

HWE and statistical power

The genotypic distribution in all *TNFAIP3* variants were found to be HWE in controls. Considering a design of unmatched case-control, a hypothesis of gene only, a ratio of 1.06 control per case, the frequency of *TNFAIP3* rs2230926G minor allele in controls (2.3%), a dominant model, the prevalence of RA reported in Mexico (1.6%), and an OR of 1.5, our study had a statistical power of 83.7%.

Association analysis of TNFAIP3 SNVs in cases and controls

The allelic and genotypic frequencies of the functional *TNFAIP3* rs2230926T/G variant in both cases and controls, were similar under all genetic models. Thus, this SNV was not associated with RA (Table 1). Because rs2230926T/G was in almost complete LD (r^2 = 0.98) with rs2230924T/G, there was an identical result between both variants. We also

observed similar allelic and genotypic frequencies in the other six *TNFAIP3* variants among both groups (Table 1). Therefore, we did not identify association between *TNFAIP3* variants and RA susceptibility in Mexican patients under any genetic model (Table 1).

Allelic frequency of TNFAIP3 rs2230926T/G, rs6920220G/A, and rs10499194C/T *in other ethnic groups*

Allelic frequencies and associations of the *TNFAIP3* rs2230926T/G, rs6920220G/A and rs10499194C/T variants in patients with RA and controls have been studied extensively in Caucasians and Asians. We found some minor differences in allelic frequencies of the *TNFAIP3* rs6920220G/A and rs10499194C/T variants in our controls compared to Caucasian and Asian controls (Table 2).

Haplotypes and LD

Haplotype analysis identified ten different allele combinations of the eight *TNFAIP3* variants. After analysis, we did not report any association between these haplotypes and RA (data not shown). On the other hand, there was an almost complete LD (r^2 ≈0.98) between *TNFAIP3* rs5029924C/T and rs2230926T/G, and between rs61593413T/A and rs59693083 (r^2≈0.98), whereas the remaining *TNFAIP3* variants were not in LD (Figure 2).

Discussion

The present study was undertaken to examine in detail the possible association of eight variants of *TNFAIP3* that encodes the A20 protein, a negative regulator of NF-κB, in a cohort of Mexican patients with RA. These SNVs were selected because seven of them are in the *TNFAIP3* promoter, in proximity of potential transcription factor-binding sites and because two of them have been associated with RA in Caucasian and Asian-derived populations [14, 15]. The other is a non-synonymous variant that appears to produce decreased enzymatic activity [16]. Although some of these *TNAFIP3* SNVs have been found in association to RA or SLE in Asians and Caucasians [14, 15, 22, 23], none of them was associated to RA in our population, indicating that, at least in Mexicans, these variants may not be primarily involved in the pathogenesis of this AD.

The A20 protein, product of the *TNFAIP3* gene, is a negative regulator of NF-κB in response to TNF, IL-1, IL-17, and other cytokines. A20 limits the release and translocation of NF-κB to the cell nucleus by means of deubiquitylation-ubiquitylation of some components of the I-κB-phosphorylation pathway, which is of major importance to prevent uncontrolled inflammation. A20-deficient mice die prematurely from multi-organ inflammation due to increased NF-κB signaling [10]. In addition, A20-deficient mouse embryonic fibroblasts have increased I-κBα degradation and persistent NF-κB signaling [10]. Thus, decreased function of A20 leads to an enhanced response to pro-inflammatory cytokines, increased inflammation in transgenic models and chronic inflammatory conditions [24-28].

Previous GWAS have found association of the *TNFAIP3* rs6920220G/A and rs10499194C/T variants with RA [11, 29, 30, 31], meanwhile, some candidate gene studies and a fine-mapping at 6q23 have replicated this finding, in addition to the rs2230926T/G SNV, primarily in Asian and European-derived populations [22, 32-35]. However, other GWAS or candidate gene studies have not reproduced the associations of these or other *TNFAIP3* variants in Latin-Americans, Asians, and Europeans [36-39]. Thus, associations of these variants with RA are not universal. Moreover, these variants have yet to be evaluated in highly admixed populations, such as Mexicans.

Our data show that none of eight *TNFAIP3* variants are associated with RA in Mexican patients. Similar findings have been reported in Spaniards, Slovaks, and Chileans [36-39]. Indeed, two recent meta-analyses identified *TNFAIP3* rs6020220G/A and rs10499194C/T as risk and protection factors for RA in Caucasian patients, respectively, but not in Asians [14, 15]. These contrasting results suggest that *TNFAIP3* variant association with RA may depend on additional genes present in the genetic background of different ethnic groups.

It was of interest that the frequency of the *TNFAIP3* rs6920220A minor allele in our controls was lower than in Caucasians, where it has been associated with RA (Table 2). On the other hand, the *TNFAIP3* rs10499194T minor allele, which appears to be protective for RA in Caucasians [15], had a higher allele frequency in our controls than in Caucasians, Asians and Mexicans included in the 1000 Genome Project (Table 2). Therefore, the genetic composition of the Mexican people differs slightly from other populations, possibly affecting their susceptibility to RA and other AD.

On the other hand, the amino acid change of the *TNFAIP3* rs2230926T/G (Phe127Cys) variant, which has been associated with RA susceptibility in Asians, affects the TNF-induced NF-κB signaling [16]. The Cys127 variant produces a modest, but consistently lower inhibition of TNF-dependent NF-κB activity than Phe127. Several studies in Caucasian, Asian, and African-derived populations have reported that Cys127 *TNFAIP3* is associated with susceptibility to RA [14,22,23,40,41]. However, one meta-analysis found that it is a risk factor for RA only in Asians [14]. As certain genetic associations can only be identified when different alleles combine to form haplotypes, we examined whether different *TNFAIP3* haplotypes were associated with RA. When we examined this variant with different combinations of the seven *TNFAIP3* SNVs in patients with RA, the results were again negative. Our data suggest that *TNFAIP3* rs2230936T/G (or combined with the other seven variants) is not a risk factor to RA in Mexicans.

TNFAIP3 rs373421182G/C, rs139054966T/G, rs5029924C/T, rs59693083A/G, and rs61593413T/A SNVs were not associated with RA in Mexicans. As far as we know, none of these variants had been previously examined in any population with RA.

Finally, although we did a preliminary screening of the possible association between the ACPAs positivity with the different minor alleles of the eight *TNFAIP3* variants in some of patients with RA, our data suggest that none of these are distinctly associated with ACPA positive RA (data not shown). However, the low sample size (n=69) examined does not permit to draw any conclusion. In a near future, we will expand the ACPA screening to include most of our RA cohort to examine in detail association with seropositive RA, which is a distinct entity and deserves a separate analysis [40,41].

RA is a multifactorial disease with contribution of many different genes in its pathogenesis. *TNAFIP3* is only one of them and it seems clear that its contribution is relatively weak compared to other autoimmune diseases. In conclusion, *TNFAIP3* gene variants do not appear to contribute to the pathogenesis of RA in Mexicans, as opposed to other populations where some *TNFAIP3* variants play a role in the pathogenesis of RA in the presence of additional susceptibility genes.

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Authors' contribution

PhD. ISA-U and PhD JR-B implemented the experiments, also participated with the data analysis, and contributed to the writing of the manuscript. M.D. REB-C participated with the recruitment of patients with RA and SLE. PhD. GV-A implemented the experiments. PhD. JM was responsible for the conception and design of the experiments and contributed to the writing of the manuscript.

Declarations

Conflict of interest

The authors declare that there are no personal or financial conflicts of interest regarding the present study.

Ethical approval

This study was conducted according to the Declaration of Helsinki and approved by the Research and Bioethics Committee of HJM (Registry Number HJM-0446/18-I).

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Figures

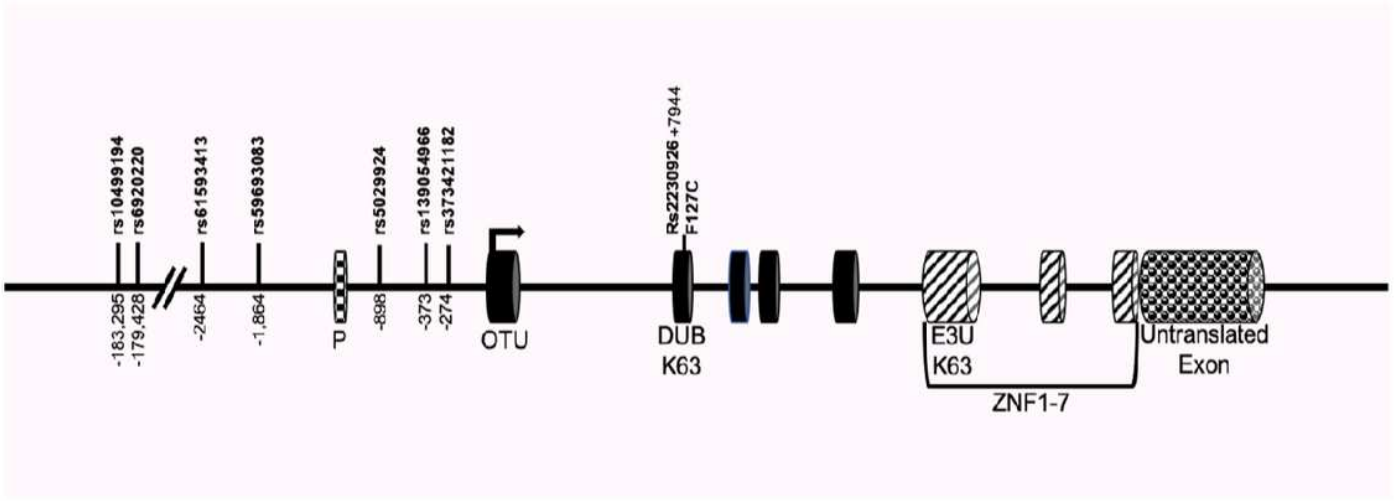

Figure 1

Positions of the TNFAIP3 SNVs evaluated in this study.

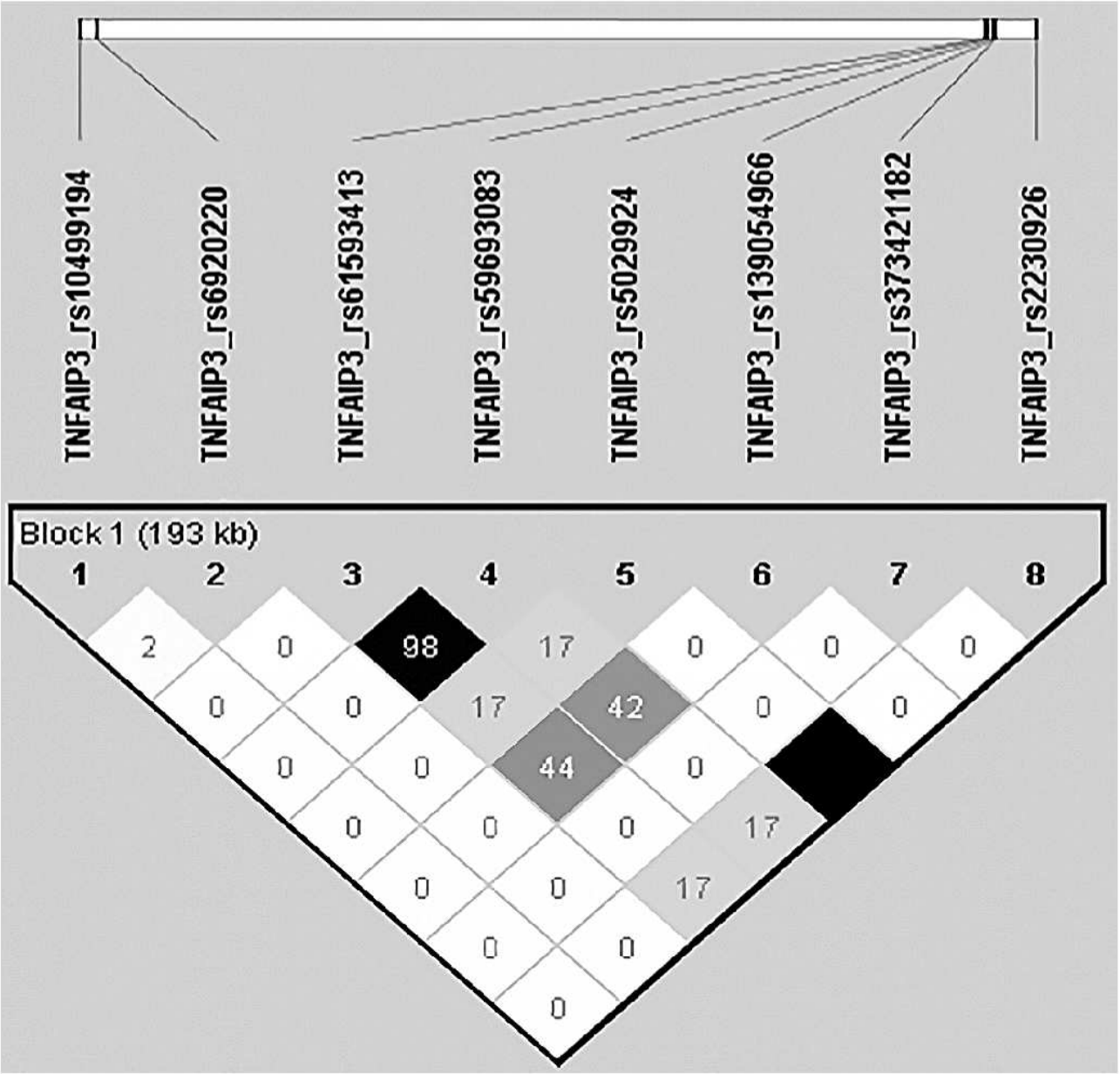

Figure 2

Complete LD between two TNFAIP3 SNVs in RA patients and controls

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