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ORIGINAL ARTICLE

STAT4 rs7574865 G/T polymorphism is associated with rheumatoid arthritis and disease activity, but not with anti-CCP antibody levels in a Mexican population

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Abstract Rheumatoid arthritis (RA) is a systemic autoimmune disease in whose etiology genetic factors are known to play an important role. Among the genes associated with RA, *STAT4* could be an important factor in conducting helper T cells toward the pro-inflammatory Th1 and Th17 lineages. The aim of this study is to determine the association of the *STAT4* polymorphism rs7574865 with RA, disease activity, and anti-cyclic citrullinated peptide (CCP) antibody levels in a Mexican population. Genotyping was carried out using the Taqman® system from Applied Biosystems in 140 patients with RA and 150 healthy subjects. Disease activity was evaluated by a rheumatologist using the DAS28 and Spanish-HAQ-DI instruments. Anti-CCP levels were determined by ELISA. Associations of the genotypes of rs7574865 with DAS28, HAQ, and anti-CCP antibody levels with RA were determined. Findings showed that the GT and TT genotypes and the T allele from rs7574865 were all associated as risk factors for RA, independently of their anti-CCP status. An association with moderate-to-high disease activity (DAS28 ≥ 3.2) was also found. Additionally, patients with the GT or TT genotypes showed lower HAQ values than those who carried the GG genotype. No differences in anti-CCP antibody levels or DAS28 and genotypes were found. This work supports the association of the *STAT4* rs7574865 polymorphism with RA and disease activity, but not with anti-CCP antibody levels in a Mexican population.

Keywords Biomarkers · Case–control study · Polymorphism · Rheumatoid arthritis · SNP

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by articular, bone and cartilage destruction, and synovial hyperplasia [1]. Though its etiology remains unclear, it has been suggested that both environmental and genetic factors may be involved [2]. In Mexico, the prevalence of RA has been estimated at 1.6 % of the general population [3]. RA is characterized by the production of two antibodies: the rheumatoid factor (FR) and anti-cyclic citrullinated peptides (anti-CCPs) [4]. The latter are present in about two thirds of all RA patients [5]. RA is also a chronic inflammatory and destructive disease in which numerous cytokines act through both cascades of action and redundancy [4].

The impact of genetic factors compared to environmental ones is reflected by the 15–30 % concordance rates of RA in monozygotic twins. Moreover, it has been shown that up to 60 % of disease susceptibility is due to genetic factors [6], such as gene polymorphisms in *HLA-DR*, the protein tyrosine phosphatase nonreceptor type 22 (*PTPN22*), and peptidylarginine deiminase type 4 (*PADI4*), both of which have been associated with RA [4].

STAT4 encodes the signal transducer and activator of transcription 4 (STAT-4), a member of the STAT protein family [7] that is expressed in monocytes, dendritic cells, and macrophages at inflamed sites [8]. Activation of STAT-4 is dependent on interleukin (IL)-12 and its receptor, which play essential

roles in Th1 cell differentiation and proliferation [9]. Moreover, STAT-4 transduces signals that promote the expression of IL-23, a key cytokine involved in Th17 expansion [1, 10, 11]. Therefore, STAT-4 is considered an important player that directs helper T cells toward the pro-inflammatory Th1 and Th17 lineages [11]. Identification of *STAT4* as a genetic risk factor for RA emerged from the model of proteoglycan-induced arthritis in mice, in which *STAT4*-deficient subjects displayed less disease and lower inflammation parameters compared to the wild-type mice used as controls [12]. Since Th1 and Th17 cells play an important role in chronic inflammatory disorders and *STAT4* is considered a key molecule in both of these lineages, it may play a crucial role in the development of autoimmune inflammatory diseases such as RA [12].

An SNP has been described in the third intron of the *STAT4* gene, namely, rs7574865, a G/T change in Chr.2:191964633 [11]. Although the functional effect of this SNP is unclear, splicing variation effects have been suggested [13]. Moreover, the association of the *STAT4* polymorphism rs7574865 with RA has been described for Korean [2], Japanese [7], Caucasian, Chinese [8], Algerian [14], and Egyptian populations [15]. On the other hand, the possible association of rs7574864 with DAS28 scores remains controversial. Shen et al., for example, reported an association of the GT genotype with DAS28 > 3.2 [12], but Setting et al. found no such association [15] in Chinese and Egyptian sample populations, respectively. It is important to note that no association studies of this polymorphism have yet been reported for Mexico.

Considering that allelic frequencies of genes frequently differ from one population to another, ethnic-specific association studies are required to confirm genetic associations in different population groups. Thus, the aim of this study was to determine the association of the *STAT4* polymorphism rs7574865 with RA, disease activity, and anti-CCP levels in a population from western Mexico.

Materials and methods

Subjects

A total of 140 unrelated RA patients, regardless of onset and disease duration, and diagnosed according to the ACR/EULAR 2010 criteria [16] at IMSS General Hospital No. 1 in Tepic Nayarit, Mexico, were included in this study. Evaluation of Disease Activity Scores using 28-joint counts (DAS28) and the Spanish version of the Health Assessment Questionnaire Disability Index (Spanish-HAQ-DI) [17] were applied to the RA patients by a rheumatologist. Also, 150 clinically healthy subjects were recruited as a control group. All subjects were Mexican residents from Nayarit, and all gave their informed consent prior to inclusion in the study, according to the stipulations of the 1964 Helsinki Declaration and its later amendments. The study was approved by the local ethics committee.

Genotyping the rs7574865 of *STAT4*

Genomic DNA was extracted from peripheral blood following the Miller method [18]. Samples were genotyped for the rs7574865 G/T polymorphism using Taqman® allelic discrimination assay technology with a predesigned SNP genotyping assay provided by Applied Biosystems (part number C-29882391-10, Foster City, CA, USA), following the manufacturer's instructions.

Anti-CCP antibody levels

IgG anti-CCP levels were quantified by enzyme-linked immunosorbent assay (ELISA) (DRG, EIA-5653). The cutoff value (≥10 U/mL) was set according to the manufacturer's instructions. Sera with anti-CCP levels above the highest standard sample (>500 U/mL) were reassessed after appropriate dilution.

Statistical analysis

Sample size was calculated using Epidat software and the allelic frequencies from a pilot protocol, considering OR = 2.0, 95 % confidence, and 80 % power. The statistical limits were validated using the WinEpi tool (http://www.winepi.net/).

The Hardy-Weinberg equilibrium was tested using the chi-squared test. The genotype and allele frequencies of SNP rs7574865 were obtained by direct count. Differences in genotype, allele frequencies, DAS28, Spanish-HAQ-DI, and anti-CCP antibody levels were compared using chi-squared or Mann–Whitney tests using Minitab v14 software. The odds ratios (ORs) and their 95 % confidence intervals (95 % CIs) were calculated to estimate the effect of different alleles using the WinEpi tool.

Table 1 Clinical and demographic characteristics of the study population

	RA patients	Healthy controls
Age	53.17 ± 13.76	51.17 ± 12.42
Women/men	138/16	134/16
DAS28	4.34 ± 1.27	–
Anti-CCP (U/mL)	240.21 ± 423.63	–
Positive (*n*)	102	
Negative (*n*)	38	
Spanish-HAQ-DI	0.91 ± 0.50	–

RA rheumatoid arthritis, *DAS28* Disease Activity Score using 28-joint counts, *Spanish-HAQ-DI* Spanish version of the Health Assessment Questionnaire Disability Index, *Anti-CCP* IgG anti-cyclic citrullinated peptides antibodies

Table 2 Genetic model, genotype, and allele frequencies of the rs7574865 polymorphism

Model	RA ($n=140$)	Controls ($n=150$)	OR	95 % CI	p value
Codominant					
GG	34 (24.3 %)	63 (42 %)	Reference		
GT	78 (55.7 %)	66 (44 %)	2.1898	(1.2883–3.7222)	0.0035*
TT	28 (20.0 %)	21 (14 %)	2.4706	(1.2233–4.9897)	0.0108*
Dominant					
GG	34 (24.3 %)	63 (42 %)	2.2576	(1.3634–3.7384)	0.0014*
GT+TT	106 (75.7 %)	87 (58 %)			
Recessive					
GT+GG	112 (80.0 %)	129 (83.8 %)	1.5357	(0.8263–2.8544)	0.173
TT	28 (20.0 %)	21 (16.2 %)			
Allele					
G	146 (51.7 %)	192 (64)	1.6317	(1.1701–2.2753)	0.0038*
T	134 (48.3 %)	108 (36)			

RA rheumatoid arthritis, *OR* odds ratio, *CI* confidence interval

*$p<0.05$

Results

Age, the ratio of women to men, DAS28, and the results of the Spanish-HAQ-DI from the groups of patients and controls included in the study are shown in Table 1.

The genotype frequencies found for *STAT4* rs7574865 agreed with the Hardy-Weinberg equilibrium in both patients and controls ($p=0.1240$ and 0.5804, respectively). As shown in Table 2, the GT and TT genotypes showed a statistical association with RA as a risk factor, compared to the control group (OR = 2.1898, 95 % CI = 1.2883–3.7222; OR = 2. 4706, 95 % CI = 1.2233–4.9897, respectively). Analysis of the dominant model (GT+TT vs GG) showed that carrying the T allele was also associated with RA as a risk factor for the disease (OR = 2. 2576, 95 % CI = 1.3634–3.7384). Moreover, the comparison of the T and G alleles revealed a significant association with RA in this population from western Mexico (OR = 1. 6317, 95 % CI = 1.1701–2.2753). In contrast, the recessive model (TT vs GT+GG) showed no association with RA (OR = 1.5357, 95 % CI = 0.8263–2.8544).

Upon dividing the RA patients according to disease activity into low/remission (DAS28 < 3.2) and moderate/high (DAS28 ≥ 3.2) subgroups, we found that both genotypes—GT (OR = 2.5852, 95 % CI = 1.4450–4.6251) and TT (OR = 2.8750, 95 % CI = 1.3504–6.1208)—were associated only with moderate/high disease activity (Table 3).

On the other hand, of the 140 RA patients, 102 were positive and 38 negative for anti-CCP. A high variation in antibody levels was found (GG 319.7 ± 409.7; GT 207.6 ± 438.6, TT 238.7 ± 397.9), but no differences in anti-CCP antibody levels were observed ($p=0.446$). Additionally, we found that the association of rs7574865 with RA is independent of the presence of anti-CCP antibodies.

We also found that the GT and TT genotypes had lower Spanish-HAQ-DI values ($p=0.0405$ and 0.0394, respectively) than the GG genotype (reference), but that this did not occur with respect to anti-CCP levels ($p=0.611$) in anti-CCP-positive RA patients (Table 5).

Discussion

The genetic basis of RA is a field that has been explored deeply in recent years in an effort to describe genetic

Table 3 Association of genotypes from rs7574865 of *STAT4* with low/remission (DAS28 < 3.20) versus moderate/high (DAS28 ≥ 3.20) disease activity

DAS28	Genotypes (cases/controls)			OR (95 % CI)			
	GG	GT	TT	GT	p	TT	p
<3.20 ($n=28$)	10/63	13/66	5/21	1.2409 (0.5077–3.0332)	0.490	1.5000 (0.4601–4.8898)	0.413
≥3.20 ($n=112$)	24/63	65/66	23/21	2.5852 (1.4450–4.6251)	0.001*	2.8750 (1.3504, 6.1208)	0.006*

Genotype GG was used as reference

*$p<0.05$

Table 4 Association of *STAT4* rs7574865 polymorphism with RA according to the anti-CCP status

Genetic model	Anti-CCP (+) vs controls ($n = 102$) OR (95 % CI)	p value	Anti-CCP (−) vs controls ($n = 38$) OR (95 % CI)	p value	Anti-CCP (+) vs anti-CCP (−) OR (95 % CI)	p value
GT vs GG	1.8409 (1.0387–3.2627)	0.0357*	4.7727 (1.7204–13.2407)	0.0013*	0.3857 (0.1332–1.1168)	0.0723
TT vs GG	2.1429 (1.0051–4.5687)	0.0465*	N.D.	N.D.	0.4464 (0.1271–1.5677)	0.2022
(GT+TT) vs GG	1.9138 (1.1125–3.2921)	0.0183*	4.7793 (1.7672–12.9257)	0.0009*	0.4004 (0.1421–1.1287)	0.0764
TT vs (GT+GG)	1.4983 (0.7651–2.9339)	0.2365	1.6381 (0.6620–4.0536)	0.2823	0.9146 (0.3643–2.2961)	0.8493
T vs G	1.5192 (1.0573–2.1828)	0.0234*	2.0825 (1.2519–3.4644)	0.0043*	0.7295 (0.4301–1.2374)	0.2413

Anti-CCP anti-cyclic citrullinated peptide, *RA* rheumatoid arthritis, *OR* odds ratio, *CI* confidence interval, *N.D.* not determined: statistical limits were not valid

* $p < 0.05$

biomarkers that can be used to determine susceptibility and disease outcomes. Numerous studies have tested the association between various candidate genes and the development of RA in patients of distinct ethnic origin [15]. STAT-4 is a signal transducer that participates in the production of IL-23, a key cytokine involved in the differentiation of T cells into Th17 cells [19]. Given the current evidence of Th17 involvement in the chronic inflammation characteristic of RA, it would be expected that the *STAT4* risk alleles would generally enhance a Th17 response [2]. Moreover, *STAT4* is abundantly expressed in synovial macrophages in patients with RA and, it has been suggested, plays a key regulatory role in the pathogenesis and manifestation of the disease [12]. Therefore, polymorphisms of the gene of this molecule are important candidates for studies related to RA.

Our results showed that the allele T of the rs7574865 polymorphism is indeed a risk factor for RA, as both the heterozygotic (TT) and homozygotic (GT) expressions showed a statistical association with this illness. Moreover, the dominant model also showed that the T allele is one of the risk factors for the development RA in this population from western Mexico. Finally, a recently reported broad genome association study (GWAS) documented that the T allele of this polymorphism is significantly associated with RA [13].

It is important to note that the distribution of genotypes found in this work differs from those reported for Caucasian [20] and Colombian [21] populations, where the GG genotype was the one most frequently encountered. In our case, the GT genotype was the most frequent one, followed by GG and TT. These proportions are similar to those reported for a Japanese population [7]. Our findings confirm both the presence of genetic variation among ethnic groups and the importance of describing the association of SNP with RA in different populations. Likewise, the most frequent allele in a dominant inheritance model was T, as has been reported previously [2, 7, 13, 15, 20, 21].

On the other hand, the OR for the codominant (OR = 2.2851 and 2.5455) and dominant models (OR = 2.3480) obtained in our study are higher than those reported previously for some groups, including a recent meta-analysis [2, 7, 21], but similar to those found in Korean and Egyptian populations [15, 20]. In a study relevant to our results, Burgos-Vargas et al. reported that overall RA prevalence in Mexican populations is 1.6 %, compared to 1.0 % reported for other countries, and that Mexican RA patients develop the disease almost 12 years earlier than Canadians [3]. These data reveal the importance of describing genetic markers for autoimmune diseases such as RA in the Mexican population, as they could be used in the future to analyze the course of the disease.

Table 5 Clinical data in anti-CCP positive RA patients

	GG ($n = 33$)	GT ($n = 79$)	TT ($n = 28$)
Female/male	31/2	67/12	24/4
Age (M ± SD)	53.4 ± 12.8	53.01 ± 14.0	52.03 ± 15.0
DAS28 (M ± SD)	4.37 ± 1.38	4.31 ± 1.26	4.41 ± 1.26
HAQ (M ± SD)	1.11 ± 0.56	0.89 ± 0.5[a]	0.77 ± 0.42*
Anti-CCP+, no. (%)	28 (27.4)	54 (53.0)	20 (19.6)
Anti-CCP (M ± SD)	319.66 ± 409.67	207.59 ± 438.62	238.65 ± 397.92

M ± SD mean ± standard deviation, *DAS28* RA disease activity score 28, *anti-CCP* anti-cyclic citrullinated peptide antibody, *HAQ* Health assessment questionnaire

*$p < 0.05$

As shown in Table 3, we found that the GT and TT genotypes are associated with moderate/high disease activity (DAS28 ≥ 3.2). This result agrees with data reported by Shen et al. [12] who also found that the GT genotype is associated with moderate/high disease activity.

Anti-CCP antibody levels in the RA patients were found to be highly heterogeneous, as has been reported for other groups [22, 23]. In light of the fact that antibody levels have been used as predictors of RA development and progression [24], we tested the possible association of anti-CCP antibody levels (as clinical markers) with the genotypes of rs7574865, but found no significant differences. Coinciding with data reported by Elshazli et al. [25], we found that the association of the GT and TT genotypes, the T allele, and the dominant model (GT+TT) of rs7574865 with RA may not depend on the presence of anti-CCP antibodies (Table 4). This finding agrees with data reported for Dutch, Spanish, and Swedish cohorts [6, 26]. However, the effect of rs7574865 as a risk factor for the development of anti-CCP antibodies in RA in patients remains controversial. Hamad et al. have reported the association of rs7574865 in anti-CCP negative RA patients [27], but this has also been found only in anti-CCP-positive RA patients [20, 28, 29].

Additionally, no significant association of the genotypes of rs7574865 were found when our anti-CCP(+) and anti-CCP(−) groups were compared, although this result must be considered as preliminary because of the amount of data included in the analysis.

On the other hand, we found that RA patients who carry the T allele of rs7574865 (GT and TT genotypes) have lower Spanish-HAQ-DI levels than those who have the GG genotype (Table 5). These data agree with the findings reported by Setting et al. for an Egyptian population [15].

Unfortunately, the functional variant in *STAT4* that is responsible for increased disease susceptibility remains unknown. Since the susceptibility haplotype is located inside the intron 3 of *STAT4*, it is considered responsible for the splicing variation or regulatory effects of STAT-4 [7]. Although it has been reported that the expression of STAT-4 is significantly higher in the TT genotype than in genotypes GG or GT [30], it is necessary to investigate the functional effect of this SNP on *STAT4*.

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Compliance with ethical standards

Disclosures None.

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