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ORIGINAL PAPER

The −319C/+49G/CT60G Haplotype of *CTLA-4* Gene Confers Susceptibility to Rheumatoid Arthritis in Mexican Population

Norma Torres-Carrillo · Heriberto Ontiveros-Mercado · Nora Magdalena Torres-Carrillo · Isela Parra-Rojas · Héctor Rangel-Villalobos · María Guadalupe Ramírez-Dueñas · Sergio Ramón Gutiérrez-Ureña · Yeminia Valle · José Francisco Muñoz-Valle

Abstract Several single nucleotide polymorphisms (SNPs) within the *CTLA-4* gene and elevated serum levels of soluble CTLA-4 (sCTLA-4) have been associated with autoimmunity including rheumatoid arthritis (RA). In this case–control study, we evaluated the relationship between the −319C/T (rs5742909) and CT60 G/A (rs3087243) SNPs and sCTLA-4 levels in 200 RA patients and 200 control subjects (CS) from Western Mexico. Both SNPs were genotyped with the polymerase chain reaction–restriction fragment length polymorphism technique and the sCTLA-4 levels were quantified using an enzyme-linked immunosorbent assay kit. In addition, we performed a haplotype analysis, including our previous data of the +49A/G (rs231775) SNP. The G/A genotype of the rs3087243 SNP was associated with a decreased risk of RA [odd ratio (OR) 0.61, 95 % confidence interval (CI) 0.38–0.96, $p = 0.024$]. This protection was also observed in the negative anti-cyclic citrullinated peptide group of RA carriers of the A allele (OR 0.48, 95 % CI 0.22–1.05, $p = 0.042$). On the contrary, we identified the −319C/+49G/CT60G haplotype of *CTLA-4* gene as a risk factor for RA (OR 1.69, 95 % CI 1.13–2.52, $p = 0.01$). The sCTLA-4 levels were not associated with RA ($p = 0.377$), but were correlated with the functional disability of these patients ($r = 0.282$, $p = 0.012$). However, in CS the C/T genotype of the rs5742909 SNP, as well as the G/G and G/A genotypes of the rs3087243 SNP were associated with higher sCTLA-4 levels ($p < 0.001$). In conclusion, our results suggest that the −319C/+49G/CT60G haplotype of *CTLA-4* gene is a genetic marker of susceptibility to RA in Western Mexico, whereas the rs3087243 SNP confers protection against this disease. Moreover, both SNPs showed an effect on the sCTLA-4 production in our control population. However, further studies are required to evaluate the role of sCTLA-4 in RA, as well as the molecular and functional basis of the association between both *CTLA-4* gene SNPs and soluble levels of CTLA-4 in CS.

Keywords *CTLA-4* · Polymorphisms · sCTLA-4 serum levels · Rheumatoid arthritis

N. Torres-Carrillo · H. Ontiveros-Mercado · N. M. Torres-Carrillo · Y. Valle · J. F. Muñoz-Valle (✉)
Grupo de Inmunogenética Funcional, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Insurgentes 244-1, Colonia Lomas de Atemajac, Zapopan, Guadalajara C.P. 45178, Jalisco, Mexico
e-mail: biologiamolecular@hotmail.com

N. M. Torres-Carrillo
Departamento de Investigación Básica, Instituto Nacional de Geriatría, Secretaría de Salud, Mexico, D.F., Mexico

I. Parra-Rojas
Unidad Académica de Ciencias Químico Biológicas, Universidad Autónoma de Guerrero, Chilpancingo, Guerrero, Mexico

H. Rangel-Villalobos
Instituto de Investigación en Genética Molecular, Centro Universitario de la Ciénega, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico

M. G. Ramírez-Dueñas
Laboratorio de Inmunología, Departamento de Fisiología, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico

S. R. Gutiérrez-Ureña
Servicio de Reumatología, O.P.D. Hospital Civil de Guadalajara "Fray Antonio Alcalde", Guadalajara, Jalisco, Mexico

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects approximately 0.5–1 % of the adult population worldwide. It is characterized by chronic inflammation and erosion of the synovial joints, leading to joint deformity and loss of function, which is associated with significant disability and premature mortality [1]. The etiology of the RA to date is unknown. However, according to the main hypothesis, endogenous or environmental factors trigger the autoimmune reaction in genetically susceptible individuals, leading to the development of the disease [2]. Even though the precise RA pathogenesis remains uncertain, T cells, B cells, macrophages, neutrophils, and synovial fibroblasts are central in the mechanisms of joint inflammation and disease progression [3]. However, there is evidence that supports the hypothesis that mainly T cells play a dominant role in the RA immunopathogenesis [4] primarily due not only to the interaction with classical cells of the immune system, but also with tissue-specific cellular populations, thus contributing to autoantibody production, joint inflammation, and bone resorption [5]. Therefore, molecules involved in regulating the immune response of T cells may be the primary determinants of susceptibility for the development of autoimmune diseases, including RA.

T cell activation is positively and negatively regulated by a set of costimulatory receptors expressed on T cells as regulators of both positive and negative signals. The cytotoxic T lymphocyte-antigen 4 (CTLA-4), a type I transmembrane protein that belongs to the immunoglobulin superfamily, was first discovered as a negative regulator and has a pivotal role in terminating the T cell activation in vivo [6, 7]. Currently the mechanism by which CTLA-4 inhibits the activation of these cells is unknown, but various theories have been proposed [8]. CTLA-4 is homologue of CD28, principal positive costimulatory receptor of T cells, which interacts with B7 molecules (CD80/CD86) on the antigen-presenting cells (APCs) to induce T cells proliferation and cytokine production [9]. However, CTLA-4 also binds to the B7 molecules but with higher affinity than CD28, therefore it can compete efficiently with CD28 and downregulate T cells activation [10]. Thus, the CTLA-4-B7 interaction plays a critical role in regulating self-tolerance, and hence in the susceptibility to autoimmune diseases [11]. On the other hand, the native soluble form of CTLA-4 (sCTLA-4) generated by alternative splicing of the CTLA-4 messenger ribonucleic acid (mRNA) also corresponds to a functional molecule with ability to bind to B7 ligands [12, 13] and elevated serum levels have been associated with several autoimmune diseases, including RA [14–24]. Likewise, a new biological agent, CTLA-4-Ig, with the commercial name of "Abatacept", has demonstrated to improve signs and symptoms of RA patients [10], also suggesting an important role of sCTLA-4 in the immunopathogenesis of this disease.

Based on this crucial function of CTLA-4 in maintaining immune system homeostasis, the *CTLA-4* gene located on human chromosome 2q33, which consists of four exons and three introns, has been considered as a strong candidate of susceptibility for the development of multiple autoimmune processes [6, 11, 13, 25]. In addition, several single nucleotide polymorphisms (SNPs) within the *CTLA-4* gene including the −319C/T SNP (rs5742909) in the promoter region and the CT60 G/A SNP (rs3087243) in the 3′ untranslated region (3′UTR) of the exon 4 have been associated with a variety of autoimmune diseases [11, 26–39]. However, previous association studies, which evaluated these *CTLA-4* gene SNPs with regard to susceptibility to RA, have yielded variable and inconsistent results, some indicating association [40, 41], while others have not [42–47]. Thus, the relationship between these *CTLA-4* gene SNPs and RA remains unclear. Until now, similar association studies have not been performed in RA patients from Mexico. Therefore, the aim of this study was to evaluate the relationship between the rs5742909 and rs3087243 *CTLA-4* gene SNPs and sCTLA-4 serum levels in RA patients from Western Mexico.

Materials and Methods

Patients and Controls

All subjects were unrelated individuals from Mexican Mestizo population as defined by the National Institute of Anthropology [48]. We enrolled a total of 200 RA patients from Western Mexico, who were classified according to the American College of Rheumatology (ACR) criteria [49]. All RA patients were recruited from the Rheumatology Departments of the Hospital Civil "Fray Antonio Alcalde", Guadalajara, Jalisco, Mexico, and Hospital "Valentín Gómez Farías", ISSSTE, Zapopan, Jalisco, Mexico. The demographic, clinical, and biological characteristics of RA patients are shown in Table 1. Clinical disease activity and disability were evaluated in RA patients at the beginning of this study, in accordance with DAS28 (disease activity score using 28 joint counts) and Spanish HAQ-DI (Spanish version of the health assessment questionnaire disability index) scores, respectively, [50, 51]. In addition, 200 ethnically matched control subjects (CS) without clinical evidence of any autoimmune disease were enrolled in this study (123 women and 77 men with a mean age of 35 ± 12 years). The sample size was calculated using the Fleiss′s formula for proportions in case–control studies [52] with a statistical power of 80 %, a 95 % confidence interval (CI), and a maximum estimated odds ratio

Table 1 Demographic, clinical, and biological characteristics of RA patients

Characteristics	RA ($n = 200$)
Demographic	
Sex (women/men)	190/10
Age (years)	48 ± 14 (22–86)
Disease duration (years)	11 ± 10 (0.11–52)
Clinical	
Swollen joints (count 28)	5 ± 6 (0–24)
Painful joints (count 28)	7 ± 8 (0–28)
Patient's global assessment of disease status (0-10 VAS)	5 ± 3 (0–10)
DAS28 score	4.87 ± 1.50 (1.13–8.42)
Spanish HAQ-DI score	0.76 ± 0.67 (0–2.83)
Anti-CCP Ab positive (number anti-CCP Ab positive/total number of patients)	160/195 (82.10 %)
Anti-CCP Ab negative (number anti-CCP Ab negative/total number of patients)	35/195 (17.90 %)
RF positive (number RF positive/total number of patients)	166/178 (93.30 %)
RF negative (number RF negative/total number of patients)	12/178 (6.70 %)
Biological	
ESR (mm/h)	37.51 ± 14.16 (5–67)
CRP (mg/dL)	13.39 ± 30.42 (0.10–168.80)
Anti-CCP Ab (U/mL)	141.70 ± 150.41 (0.90–621.40)
RF (UI/mL)	367.45 ± 673.39 (5–4,280)
WBC (κ/μL)	6.79 ± 2.22 (2.23–16.60)
PLT (κ/μL)	301.29 ± 91.45 (108–638)
Drug treatment	
Steroids	42/200
Prednisone < 8.5 mg/day	25/42
DMARDs	154/200
Methotrexate	114/154
Chloroquine	99/154
Hydroxychloroquine	3/154
Azulfidine (Sulfasalazine)	46/154
Azathioprine	7/154
Leflunomide	4/154
Biologics	7/200
Etanercept	6/7
Infliximab	1/7
NSAIDs	147/200

The data are presented as the mean ± standard deviation, minimum and maximum scores

RA rheumatoid arthritis, *VAS* visual analogue scale, *DAS28* disease activity score using 28 joint counts, *Spanish HAQ-DI* Spanish version of the health assessment questionnaire disability index, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *anti-CCP* anti-cyclic citrullinated peptide antibodies, *Ab* antibody, *RF* rheumatoid factor, *WBC* white blood cells count, *PLT* platelet count, *DMARDs* disease-modifying antirheumatic drugs, *NSAIDs* non-steroidal anti-inflammatory drugs

(OR) of 2, resulting in 317 alleles for each study group for the less frequent polymorphism (rs5742909).

Ethical Considerations

RA patients and CS were included in this study after giving their written informed consent. The investigation was performed according to the ethical guidelines of the 2008 Declaration of Helsinki, and was approved by the ethical, investigation, and biosecurity committee of the University Center of Health Sciences of the University of Guadalajara, and by the ethical committee of the Hospital Civil ''Fray Antonio Alcalde''.

Laboratory Evaluation

Erythrocyte sedimentation rate (ESR) (Westergren method), white blood cell count (WBC), platelet count (PLT) (CELL-DYN 3700 Abbott Diagnostics), C-reactive protein (CRP), and rheumatoid factor (RF) (IMMAGE^{TM} Immunochemistry) were assayed in all subjects included in this study, who had an overnight fast. The presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies was determined using a commercially available second-generation enzyme-linked immunosorbent assay (ELISA) kit (Anti-CCP, DIASTAT^{TM}, Axis-Shield Diagnostics Limited, Dundee, Scotland, UK). A cut off of point of > 5 U/mL was used as a stringent criterion for anti-CCP positive.

DNA Extraction

For genotyping, venous blood samples of patients and controls were collected into EDTA-containing tubes, and the genomic DNA (gDNA) was extracted from peripheral blood leukocytes according to the Miller's salting-out method [53].

CTLA-4 Gene Polymorphisms Genotyping

The rs5742909 and rs3087243 *CTLA-4* gene SNPs were analyzed by the polymerase chain reaction–restriction fragment length polymorphism system which was slightly modified from the two previously described methods [27, 28]. The PCR amplification was performed in a total volume of 25 μL, containing 1 μg of gDNA, 3 μM of each oligonucleotide, 2.5 μM of each dNTP, $MgCl_2$ 1.5 mM for rs5742909 and 1 mM for rs3087243, 0.625 U/μL of *Taq* DNA polymerase, and supplied buffer enzyme 1X (Invitrogen Life Technologies, Carlsbad, CA, USA). The amplification conditions were as follows: 95 °C for 2 min, 30 cycles of 94 °C for 40 s, 60 °C for 45 s, 72 °C for 30 s, followed by 72 °C for 2 min for amplification of the

rs5742909 SNP and 95 °C for 2 min, 33 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, followed of 72 °C for 2 min for amplification of the rs3087243 SNP. The PCR products were digested at 37 °C for 1 h with 10 U of *Mse*I and 8 U of *Nco*I restriction enzymes (New England Biolabs, Beverly, MA, USA) for the identification of the rs5742909 and rs3087243 *CTLA-4* gene SNPs, respectively. Finally, the digested PCR products were electrophoresed on 3 % agarose gels stained by ethidium bromide for the identification of the rs5742909 SNP genotypes, and on 6 % polyacrylamide gels stained with silver for the identification of the rs3087243 SNP genotypes.

DNA Sequencing

The genotyping technique for the rs5742909 and rs3087243 *CTLA*-4 gene SNPs was confirmed by DNA sequencing of a subset of samples, randomly selected, using a DNA Genetic Analyzer ABI Prism 310 (Applied Biosystems) (data not shown).

Immunoassay of sCTLA-4

Soluble CTLA-4 levels were determined in serum of 79 RA patients and 80 CS using a commercially available ELISA kit (Human sCTLA-4 ELISA, BMS276, Bender MedSystems Diagnostic GmbH, Vienna, Austria). The assay sensitivity was 0.13 ng/mL and it was carried out accordingly to the manufacturer's instructions. To assure that the sCTLA-4 assay was not confounded by polyreactive/heterophile antibodies present in our patients/control population, we correlated the sCTLA-4 levels with the RF levels in both RA patients and CS, without finding a significant correlation ($r = 0.214$, $p = 0.077$ and $r = 0.075$, $p = 0.514$; respectively) (data not shown). This lack of correlation confirms that we are in fact measuring sCTLA-4 levels and not heterophile antibodies.

Statistical Analysis

Frequencies of genotypes and alleles, Hardy–Weinberg equilibrium (HWE) and OR with a 95 % CI were estimated using the Chi square test (χ^2) (Epi Info statistical software 3.3.2, Atlanta Georgia). Haplotype frequencies were inferred using HEMHAPFRE software [54]. Linkage disequilibrium (LD) at two loci was expressed as Lewontin′s corrected LD coefficient (D') [55]. Comparison data were evaluated by χ^2 test or the Fisher′s exact test when applicable. LD at three loci was evaluated according to Gavrilets et al. [56]. The p values were adjusted with Bonferroni correction for multiple testing (p_c) [57]. For other comparisons, we used parametric Student's t test, one-way ANOVA test, and nonparametric Mann–Whitney U test, Kruskal–Wallis test, and Spearman's correlation test (SPSS statistical software 10.0, Chicago Illinois), as mentioned in tables and figures. Results were given as mean values, standard deviation (SD), minimum and maximum scores. Differences were considered as significant at $p < 0.05$.

Results

RA Patients and CS

We studied 200 RA patients and 200 CS. At the time of the inclusion of RA patients, disease average duration was 11 ± 10 years with moderate disease activity (DAS28: 4.87 ± 1.50 score), as well as some functional disability to perform any daily activity (HAQ-DI: 0.76 ± 0.67 score). Respect to the laboratory assessment, the ESR, CRP, anti-CCP antibodies, and RF levels as well as the PLT count were significantly higher in RA patients (37.51 ± 14.16 mm/h, 13.39 ± 30.42 mg/dL, 141.70 ± 150.41 U/mL, 367.45 ± 673.39 UI/mL, and 301.29 ± 91.45 κ/μL, respectively) than CS (16.94 ± 11.90 mm/h, 1.14 ± 2.05 mg/dL, 1.37 ± 0.49 U/mL, 13.88 ± 8.67 UI/mL, and 260.16 ± 67.00 κ/μL, respectively) ($p < 0.001$). In addition, RA patients were being treated with disease-modifying antirheumatic drugs (DMARDs), mainly methotrexate and chloroquine, and non-steroidal anti-inflammatory drugs (NSAIDs).

Frequency of *CTLA-4* Gene Polymorphisms and Haplotype Analysis in Both Study Groups

The distribution of genotypic and allelic frequencies of the rs5742909 and rs3087243 *CTLA-4* gene SNPs in RA patients and CS is shown in Table 2. Both *CTLA-4* gene SNPs were in HWE in the CS group ($p > 0.05$; data not shown). The distribution of genotypic and allelic frequencies between both RA patients and CS groups did not show significant differences for rs5742909 SNP ($p > 0.05$), but for the rs3087243 SNP, we observed a marginal significant difference in the distribution of genotypic frequencies between both study groups ($p = 0.077$), with an OR 0.61 (95 % CI 0.38–0.96) for the G/A genotype, which indicates an association of the G/A genotype with a decreased risk of RA ($p = 0.024$). This significant association was also seen in the dominant genetic model realized for this polymorphism (OR 0.63, 95 % CI 0.41–0.98, $p = 0.029$), indicating that a single copy of the A allele is enough to reduce the risk of RA and that carrying two copies of this allele (A/A genotype) decreases the risk in the same magnitude. However, we did not observe a significant difference in the distribution of allelic frequencies between both study groups for this polymorphism ($p > 0.05$).

On the other hand, our research group recently found the +49A/G *CTLA-4* gene SNP (rs231775) to be associated

Table 2 Genotypic and allelic frequencies of *CTLA-4* gene polymorphisms in RA patients and CS

SNP	RA ($n = 200$) % (n)	CS ($n = 200$) % (n)	Global p	OR (95 % CI), p value
rs5742909				
Genotypes				
C/C*	91.00 (182)	90.00 (180)		1.0
C/T	8.00 (16)	10.00 (20)	NS	NS
T/T	1.00 (2)	0.00 (0)		NS
Alleles				
C*	95.00 (380)	95.00 (380)	NS	1.0
T	5.00 (20)	5.00 (20)		NS
Genetic model (Do)				
C/C*	91.00 (182)	90.00 (180)	NS	1.0
C/T+T/T	9.00 (18)	10.00 (20)		NS
rs3087243				
Genotypes				
G/G*	41.50 (83)	31.00 (62)		1.0
G/A	43.00 (86)	53.00 (106)	0.077	0.61 (0.38–0.96), 0.024
A/A	15.50 (31)	16.00 (32)		NS
Alleles				
G*	63.00 (252)	57.50 (230)	NS	1.0
A	37.00 (148)	42.50 (170)		NS
Genetic model (Do)				
G/G*	41.50 (83)	31.00 (62)	0.029	1.0
G/A+A/A	58.50 (117)	69.00 (138)		0.63 (0.41–0.98),0.029

The values are presented as frequency in percentage (%) and genotypes and alleles number (n). The frequencies comparison between groups was analyzed using Chi square test (χ^2) and Fisher's exact test when applicable. Both, rs5742909 and rs3087243 *CTLA-4* gene SNPs were in HWE in the CS group ($p = 0.599^{\text{Fisher's exact test}}$ and 0.714, respectively)

CTLA-4 citotoxic T lymphocyte-antigen 4, *SNP* single nucleotide polymorphism, rs5742909 −319C/T SNP, rs3087243 CT60 G/A SNP, *RA* rheumatoid arthritis, *CS* control subjects, *OR* odds ratio, *CI* confidence interval, *Do* dominant model, *NS* not significant

*Reference category: *HWE* Hardy–Weinberg equilibrium

with susceptibility to RA in Western Mexican population [58]. Based on this finding, it was of our interest to perform a haplotype analysis including data of this polymorphism. The LD analysis at two loci showed linkage between all possible combinations, further confirmed by LD assessment at three loci (Tables 3, 4, respectively). The maximum D' coefficient at two loci reached 90 % of linkage in the rs5742909/rs3087243 haplotype ($p < 0.001$). Regarding to D' coefficient evaluated at three loci, the maximum LD value was 50 %, observed in the −319T/+49G/CT60A haplotype ($p = 0.009$). Finally, in the haplotype frequencies comparison between both study groups, we identified the −319C/+49G/CT60G haplotype of *CTLA-4* gene as a haplotype of risk to RA (OR 1.69, 95 % CI 1.13–2.52, $p = 0.01$) (Table 5).

Demographic and Clinical Characteristics and Laboratorial Assessment According to the *CTLA-4* Gene Polymorphisms in RA Patients

Respect to the demographic and clinical characteristics and laboratorial assessment according to both *CTLA-4* gene SNPs in the RA patients group, we did not find any association between the rs5742909 SNP and the different

Table 3 Haplotypes frequencies and LD analysis at two loci of *CTLA-4* gene in CS

Haplotype	(%)	D'	p value
rs5742909/rs231775			
CA	62.00	1.00	0.716
CG	32.00	−1.00	0.049
TA	2.00	−0.36	0.167
TG	3.00	0.36	0.246
rs231775/rs3087243			
AG	40.00	−0.12	1.626×10^{-5}
AA	24.00	0.23	1.280×10^{-7}
GG	17.00	−0.23	2.825×10^{-24}
GA	19.00	0.12	5.085×10^{-12}
rs5742909/rs3087243			
CG	57.00	0.92	0.955
CA	38.00	−0.92	0.141
TG	1.00	−0.90	<0.001
TA	4.00	0.90	<0.001

The linkage disequilibrium (LD) is expressed as Lewontin's corrected LD coefficient (D') [55]. The comparison data was evaluated by Chi square test (χ^2) and Fisher's exact test when applicable

CTLA-4 citotoxic T lymphocyte-antigen 4, *CS* control subjects, *rs5742909* −319C/T SNP, *rs231775* +49A/G SNP, *rs3087243* CT60 G/A SNP

Table 4 Haplotypes frequencies and LD analysis at three loci of *CTLA-4* gene in CS

Haplotype rs5742909/ rs231775/ rs3087243	(%)	D'	p_c value
CAG	40.00	−1.00	0.325
CAA	22.00	0.20	1.000
CGG	16.00	−0.03	0.491
CGA	16.00	0.67	1.000
TAG	0.00	0.75	0.079
TAA	2.00	−0.40	0.499
TGG	1.00	0.11	1.000
TGA	2.00	−0.50	0.009

The linkage disequilibrium (LD) at three loci was evaluated according to Gavrilets et. al. [56]. The p values were adjusted with Bonferroni correction for multiple testing (p_c) [57]

CTLA-4 citotoxic T lymphocyte-antigen 4, *CS* control subjects, *rs5742909* −319C/T SNP, *rs231775* +49A/G SNP, *rs3087243* CT60 G/A SNP, D' corrected LD coefficient

Table 5 Haplotypes frequencies comparison between RA patients and CS at three loci of *CTLA-4* gene

Haplotype rs5742909/ rs231775/ rs3087243	RA % (n)	CS % (n)	OR (95 % CI), p value
CAG*	36.66 (141)	40.18 (144)	1.0
CAA	15.93 (62)	21.93 (78)	0.81 (0.54–1.22), 0.31
CGG	25.00 (96)	16.24 (58)	1.69 (1.13–2.52), 0.01
CGA	18.79 (73)	16.34 (59)	1.26 (0.84–1.91), 0.27
TAG	0.00 (0)	0.00 (0)	NA
TAA	0.00 (0)	2.42 (9)	NA
TGG	0.52 (2)	0.56 (2)	1.02 (0.14–7.35), 1.00[a]
TGA	3.11 (12)	2.32 (8)	1.53 (0.61–3.86), 0.49[a]

The values are presented as frequency in percentage (%) and haplotypes number (n). The haplotype frequencies were inferred using the HEMHAPFRE software [54]. The comparison data was evaluated by Chi square test (χ^2) and Fisher's exact test when applicable

CTLA-4 citotoxic T lymphocyte-antigen 4, *RA* rheumatoid arthritis, *CS* control subjects, *rs5742909* −319C/T SNP, *rs231775* +49A/G SNP, *rs3087243* CT60 G/A SNP, *OR* odds ratio, *CI* confidence interval, *NA* not applicable

[a] Fisher's exact test

* Reference haplotype

characteristics evaluated (data not shown). Nevertheless, the rs3087243 SNP was associated with the number of swollen joints (global $p = 0.049$), since RA carriers of the G/G genotype presented higher number of these joints than carriers of the A/A genotype ($p = 0.022$, Mann–Whitney U test). Furthermore, the G allele was associated with higher disease clinical activity ($p = 0.023$), as well as higher number of painful and swollen joints ($p = 0.020$ and 0.011, respectively) than the A allele (data not shown). In addition, we stratified RA patients according to anti-CCP antibody status, and we did not observe an association of the rs5742909 SNP with any RA patient subset. However, the A allele of the rs3087243 SNP was associated with protection for the development of RA in the negative anti-CCP group of RA patients (OR 0.48, 95 % CI 0.22–1.05, $p = 0.042$), as compared with CS. Regarding the positive anti-CCP group of RA patients, we did not find a significant association with this polymorphism ($p = 0.075$) (Table 6).

Soluble CTLA-4 Levels and Their Clinical Correlation in RA Patients

Soluble CTLA-4 was detectable in both study groups and there was no variability in the individual results. The sCTLA-4 levels were very similar in RA patients and CS without a significant difference ($p = 0.377$) (Fig. 1). Likewise, we did not observe an association between sex and age with sCTLA-4 levels in any study group, as well as sCTLA-4 levels were not correlated with the clinical disease activity (DAS28 index) in RA patients ($p > 0.05$) (data not shown). However, the sCTLA-4 levels were positively correlated with the functional disability of these patients ($r = 0.282$, $p = 0.012$) (Fig. 2).

Soluble CTLA-4 Levels According to the *CTLA-4* Gene Polymorphisms in Both Study Groups

The sCTLA-4 levels were analyzed according to both *CTLA-4* gene SNPs. In the RA patients group, we did not find significant differences in the sCTLA-4 levels with respect to both rs5742909 and rs3087243 SNPs ($p = 0.445$ and 0.552, respectively) (Fig. 3). However, in the CS group both rs5742909 and rs3087243 SNPs were significantly associated with sCTLA-4 levels, since the CS carriers of the C/T genotype of the rs5742909 SNP showed higher sCTLA-4 levels than CS carriers of the C/C genotype ($p < 0.001$), and the CS carriers of the G/G and G/A genotypes of the rs3087243 SNP showed higher sCTLA-4 levels than CS carriers of the A/A genotype ($p < 0.001$) (Fig. 4). Likewise, with respect to the alleles, the T allele of the rs5742909 SNP was associated with higher sCTLA-4 levels than the C allele, and the G allele of the rs3087243 SNP was associated with higher sCTLA-4 levels than the A allele ($p < 0.001$) (data not shown).

Table 6 Frequency of *CTLA-4* gene polymorphisms in RA patients stratified according to anti-CCP antibodies status

	rs5742909 SNP		RA versus all CS		rs3087243 SNP		RA versus all CS	
	CC % (*n*)	CT+TT % (*n*)	*p* value	OR (95 % CI)	GG % (*n*)	GA+AA % (*n*)	*p* value	OR (95 % CI)
Controls ($n = 200$)	90.00 (180)	10.00 (20)			31.00 (62)	69.00 (138)		
Anti-CCP Ab-positive[a] ($n = 160$)	92.00 (147)	8.00 (13)	0.540	0.80 (0.36–1.74)	40.00 (64)	60.00 (96)	0.075	0.67 (0.43–1.07)
Anti-CCP Ab-negative[a] ($n = 35$)	86.00 (30)	14.00 (5)	0.448	1.50 (0.45–4.67)	49.00 (17)	51.00 (18)	0.042	0.48 (0.22–1.05)

The values are presented as frequency in percentage (%) and alleles number (n). The frequencies comparison between groups was analyzed using Chi square test (χ^2)

CTLA-4 citotoxic T lymphocyte-antigen 4, *SNP* single nucleotide polymorphism, *rs5742909* −319C/T SNP, *rs3087243* CT60 G/A SNP, *RA* rheumatoid arthritis, *CS* control subjects, *OR* odds ratio, *CI* confidence interval, *anti-CCP* anti-cyclic citrullinated peptide, *Ab* antibody

[a] Referred as subgroups of RA patients

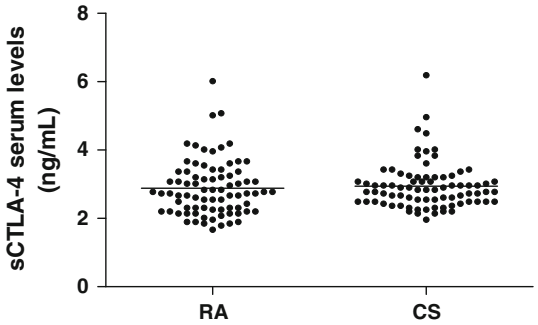

Fig. 1 Soluble CTLA-4 serum levels in RA patients and CS. The mean difference between RA patients ($n = 79$, 2.88 ± 0.81 ng/mL) and CS ($n = 80$, 2.95 ± 0.68 ng/mL) was analyzed using Mann–Whitney *U* test ($p = 0.377$). *RA* rheumatoid arthritis, *CS* control subjects, *sCTLA-4* soluble form of CTLA-4

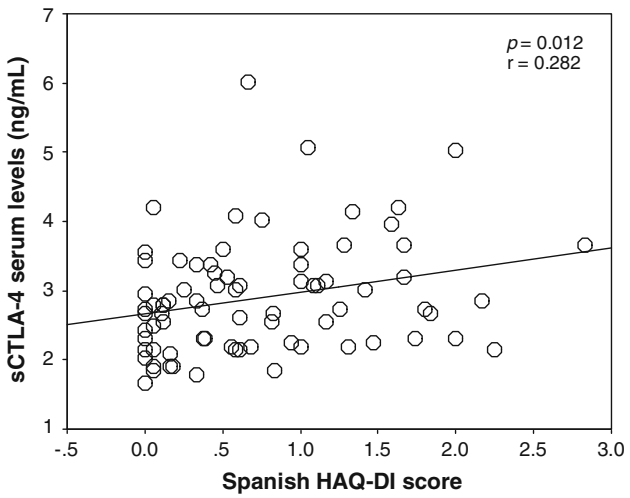

Fig. 2 Correlation between sCTLA-4 serum levels and functional disability in RA patients. The relationship between sCTLA-4 serum levels and Spanish HAQ-DI score in RA patients ($n = 79$) was analyzed using Spearman correlation test. *RA* rheumatoid arthritis, *sCTLA-4* soluble form of CTLA-4, *Spanish HAQ-DI* Spanish version of the Health Assessment Questionnaire Disability Index

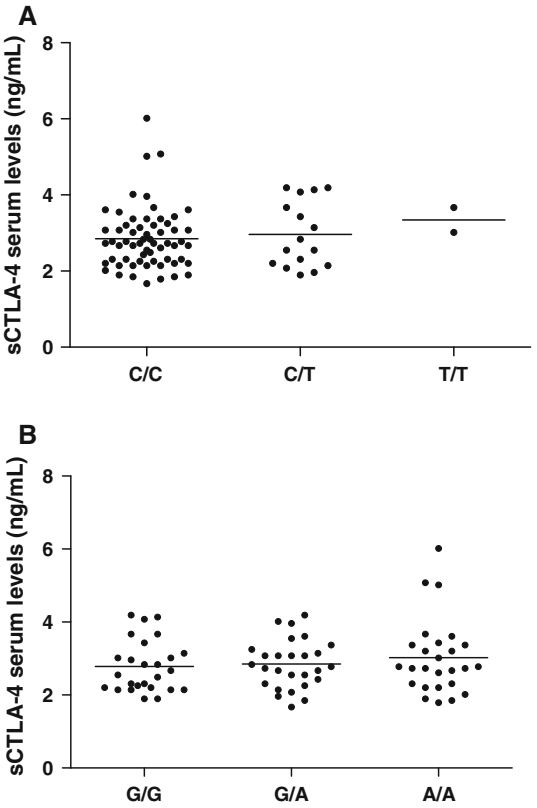

Fig. 3 Soluble CTLA-4 serum levels observed in RA patients according to the *CTLA-4* gene polymorphisms. *Panel a*. Levels of sCTLA-4 in RA patients according to rs5742909 *CTLA-4* gene SNP. The mean difference between groups was analyzed using Kruskal–Wallis test ($p = 0.445$). The sCTLA-4 serum levels observed according to each genotype were the following: C/C (2.85 ± 0.81 ng/mL), C/T (2.96 ± 0.87 ng/mL), and T/T (3.34 ± 0.46 ng/mL). *Panel b* Levels of sCTLA-4 in RA patients according to rs3087243 *CTLA-4* gene SNP. The mean difference between groups was analyzed using one-way ANOVA test ($p = 0.552$). The sCTLA-4 serum levels observed according to each genotype were the following: G/G (2.78 ± 0.71 ng/mL), G/A (2.85 ± 0.67 ng/mL), and A/A (3.02 ± 1.03 ng/mL). *RA* rheumatoid arthritis, *CTLA-4* citotoxic T lymphocyte-antigen 4, *sCTLA-4* soluble form of CTLA-4, *rs5742909* −319C/T SNP, *rs3087243* CT60 G/A SNP

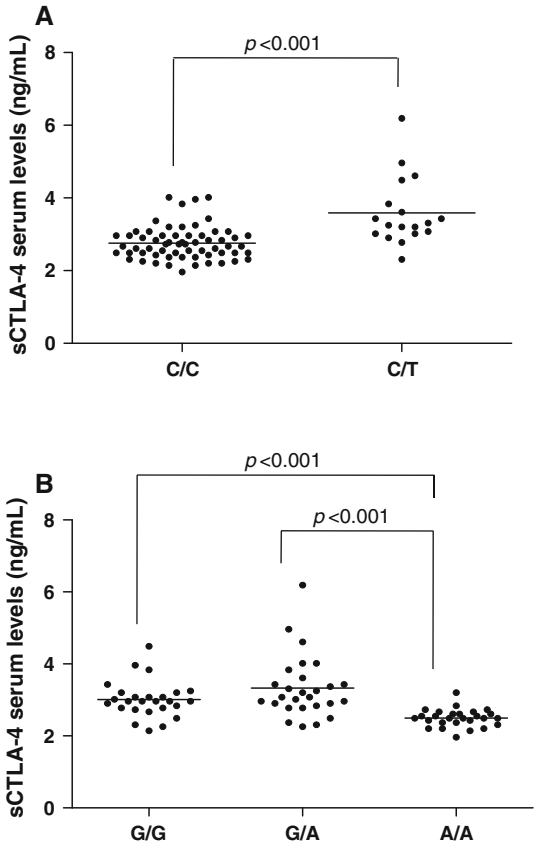

Fig. 4 Soluble CTLA-4 serum levels observed in CS according to the *CTLA-4* gene polymorphisms. *Panel a*. Levels of sCTLA-4 in CS according to rs5742909 *CTLA-4* gene SNP. The mean difference between groups was analyzed using Mann–Whitney *U* test. The sCTLA-4 serum levels observed according to each genotype were the following: C/C (2.76 $\pm$ 0.46 ng/mL) and C/T (3.59 $\pm$ 0.93 ng/mL). *Panel b*. Levels of sCTLA-4 in CS according to rs3087243 *CTLA-4* gene SNP. The mean difference between groups was analyzed using one-way ANOVA test. The sCTLA-4 serum levels observed according to each genotype were the following: G/G (3.01 $\pm$ 0.51 ng/mL), G/A (3.33 $\pm$ 0.86 ng/mL), and A/A (2.50 $\pm$ 0.25 ng/mL). *CS* control subjects, *CTLA-4* citotoxic T lymphocyte-antigen 4, *sCTLA-4* soluble form of CTLA-4, *rs5742909* −319C/T SNP, *rs3087243* CT60 G/A SNP

Discussion

Genes encoding proteins involved in the regulation, activation, and suppression of T cells have been considered as candidate genes for many autoimmune diseases. *CTLA-4* gene is one of the major susceptibility genes associated with autoimmunity and several genetic polymorphisms within this gene have been reported. One of the most studied *CTLA-4* gene polymorphisms is the rs231775 SNP, which was recently found to be associated with susceptibility to RA in Western Mexican population by our research group [58]. Based on the above, it was of our interest to also evaluate two other important polymorphisms of the *CTLA-4* gene (rs5742909 and rs3087243), and to our knowledge, this is the first study that investigates the role of these SNPs as well as the sCTLA-4 serum levels in RA patients from Mexican population.

We did not find an association between the rs5742909 SNP and RA. This is in accordance with previous reports of this SNP with RA in Spanish [42], Korean [43], and British [44] populations and like us, they also found a very low frequency of the T/T genotype. In this regard, our results together with other studies suggest that it is unlikely that the rs5742909 *CTLA-4* gene SNP has an important effect on susceptibility to RA, although it would be necessary to perform a further association study with a large number of samples, to confirm this result. Moreover, the contribution of other variants located within the promoter region of the *CTLA-4* gene in susceptibility to RA cannot be discarded.

On the other hand, the G/A genotype of the rs3087243 SNP was significantly associated with a decreased risk of RA, being the G/G genotype which confers susceptibility to this disease (OR 1.65, 95 % CI 1.04–2.61, $p = 0.024$). This protective role was also observed in the negative anti-CCP group of RA carriers of the A allele. In concordance with our results, in previous studies, the G/G genotype has been strongly associated with susceptibility to RA in Chinese [40] and North American [41] populations. In addition, we found an association between the G/G genotype and a high number of swollen joints; and between the G allele and a high clinical disease activity, as well as a high number of painful and swollen joints. Therefore, our results provide additional evidence that support the possible true-positive association of the rs3087243 *CTLA-4* gene SNP with RA.

We also observed that the three loci studied (rs5742909, rs231775, and rs3087243) are linked in this population reaching strong LD values, especially for rs5742909 and rs3087243 SNPs. Together this data suggests that these three genetic markers need to be analyzed as a panel rather than their single allele frequencies [59]. It is worth pointing out that multilocus analysis requires taking into account some corrections to minimize bias related to multiple testing. In addition, we identified the −319C/+49G/CT60G haplotype of *CTLA-4* gene as a risk haplotype, since carriers of this haplotype have 1.69 times more probability to be associated with RA. To our knowledge, there are no similar studies that evaluate the relationship between haplotypes specifically formed by these three SNPs and RA which could allow us to compare our results.

However, it is of great interest that the genetic variants of the studied polymorphisms have been previously reported as functional and associated with altered immune responses. The T allele of the rs5742909 SNP has been associated with higher promoter activity and therefore, an increased protein and gene expression (membrane CTLA-4 and CTLA-4 mRNA, respectively) and a stronger negative

regulatory effect on T cells by CTLA-4, leading to suppression of the immune response, contrary to the C allele, which presumably would be associated with a more effective immune response [60, 61].

On the other hand, the rs231775 SNP also influences T cell activation, affecting the inhibitory function of CTLA-4 since the G allele has been associated with lower protein expression than the A allele, and therefore a reduced control of the activation and a pronounced proliferation of T cells, producing an enhanced immune response that could lead to an altered T cell regulation, which might contribute to the pathogenesis of several autoimmune diseases including RA [11, 58, 61–66]. It has also been postulated that the rs231775 polymorphism in the leader sequence may influence the rates of endocytosis or surface trafficking [62], the CTLA-4 glycosylation, and the intracellular/surface partitioning, and hence, alter the inhibitory function of this molecule [67]. Likewise, the G risk allele of the rs3087243 SNP has been associated with variations in both sCTLA-4 and sCTLA-4 mRNA levels [26, 68], as well as with the levels of membrane and cytoplasmic CTLA-4 [69].

Regarding sCTLA-4 levels, we found no association between these levels and RA. This is consistent with those observed by Wan et al. [70] who also found very similar sCTLA-4 levels between RA patients and controls without a significant difference. In addition, previous reports also have not shown an association of the sCTLA-4 levels with type I diabetes and autoimmune thyroid disease [32, 71]. However, high sCTLA-4 levels have been associated with psoriasis vulgaris [19], systemic lupus erythematosus [16, 18], myasthenia gravis [15], Graves' disease, Hashimoto's thyroiditis, [14, 20, 68], autoimmune pancreatitis [21], celiac disease [22], type 1 diabetes [23], and diffuse cutaneous systemic sclerosis [17]. In Additional, unlike our results, Toussirot et al. [24] and Cao et al. [72] observed a significant association between the sCTLA-4 levels and RA.

Based on the above, the possible pathogenic role of sCTLA-4 in autoimmunity might be speculated: sCTLA-4 could inhibit the early activation of T cells by blocking CD28-CD80/CD86 interaction or conversely, high levels of sCTLA-4 could block the CTLA-4-CD80/CD86 interaction in later stages of T cells activation, causing a decrease of the inhibitory signaling and therefore, an increased T cell activation and autoreactivity [20]. Thus, the exact immunopathological role of sCTLA-4 in the development of RA may be complex and requires ongoing studies [72]. We also observed a positive correlation between the sCTLA-4 levels and functional disability of RA patients. This could indirectly suggest the participation of sCTLA-4 in the severity of RA, although this would have to be confirmed using a specific index as a severity measure in these patients.

Nonetheless, recently Cao et al. found a significant association between sCTLA-4 and RA, as well as a significant and positive correlation of the sCTLA-4 levels with disease activity measured through the DAS28 score. They also observed that immunosuppressant therapy with leflunomide, significantly decreased the sCTLA-4 levels in these patients [72]. Therefore, in our results, we cannot exclude a possible role of sCTLA-4 in the RA pathogenesis. Follow-up longitudinal studies are necessary for the sCTLA-4 levels, as well as serial correlations with the disease activity in patients with early RA recruited before any treatment is introduced, to reach a definitive conclusion.

Moreover, we also did not observe an association between both rs5742909 and rs3087243 *CTLA-4* gene SNPs and sCTLA-4 levels in the RA group. This is consistent with previous studies performed in other autoimmune diseases [16, 19, 23, 71, 73]. However, Daroszewski et al. [68] found a significant association of the G allele of the rs3087243 SNP with higher sCTLA-4 levels and only a trend of higher levels of this protein in carriers of the T allele of the rs5742909 SNP. Recently, the CTLA-4-Ig fusion protein (Abatacept) has shown to significantly reduce the disease activity and improve the physical function of RA patients [70]. In this sense, we could suggest that carriers of the genotypes which appear to associate with higher sCTLA-4 levels would probably have a better response to treatment; however, other kind of studies will be necessary to confirm this possibility.

Nevertheless, in the CS group we found a significant association of both C/T genotype and T allele of the rs5742909 SNP with higher sCTLA-4 levels. To our knowledge, there are no studies which evaluate the rs5742909 *CTLA-4* gene SNP in relation to the sCTLA-4 levels in healthy subjects. To date, there are also no reports to suggest that this SNP affects any known consensus sequence within the promoter region of the *CTLA-4* gene. However, the T allele was previously associated with higher transcriptional activity than the C allele [60]. Thus, it has been reported that healthy carriers of the T allele show a high expression of both membrane CTLA-4 and mRNA that encodes to membrane CTLA-4 [61]. Our results together with those mentioned earlier support the hypothesis that the presence of the T allele (which contributes to a higher *CTLA-4* gene expression and consequently represents one mechanism to inhibit the exaggerated immune activity) could be considered as protective for autoimmune processes by the reduction of the risk for the disease.

The G/G and G/A genotypes as well as the G allele of the rs3087243 SNP were also associated with higher sCTLA-4 levels in CS. These results are contradictory with other publications which reported a lack of association between this SNP and sCTLA-4 in two independent control populations [32, 71]. However, previously Ueda et al. [26] found that the G risk allele is associated with low levels of

the sCTLA-4 mRNA in healthy subjects, suggesting that this SNP could determine the efficiency of the splicing and the sCTLA-4 mRNA production. Therefore, we could confer that carriers of the risk allele are immunologically less protected and consequently have a greater risk of developing any autoimmune condition, since these subjects at protein level could possibly have lower sCTLA-4 levels.

Even though low-sCTLA-4 mRNA levels are present in susceptible individuals [26], high levels of this protein have been observed in serum of subjects with autoimmune disease [14–24, 72]. The possible explanation for this discrepancy may be that there is no direct relationship between mRNA levels at the cellular level and circulating protein in serum. In this regard, elevated sCTLA-4 levels may simply be due to increased half-life and/or decreased turnover of protein despite increased levels of synthesis. Also, it is possible that low-sCTLA-4 mRNA levels reflect a feedback regulatory loop in which the mRNA levels are reduced in the presence of higher levels of the sCTLA-4 protein [73]. Furthermore, it is known that translational regulatory elements at the 3′UTR have an important role in several mechanisms of translational control in a large number of mRNAs [74]. Mutations within these 3′UTR sequences might disrupt the translational regulation process and cause changes in the translational levels of individual mRNAs in both physiological and pathological conditions [75].

In conclusion, our results suggest that the −319C/+49G/CT60G haplotype of *CTLA-4* gene is a genetic marker of susceptibility to RA in Western Mexico, whereas the rs3087243 SNP confers protection against this disease. Moreover, both SNPs showed an effect on the sCTLA-4 production in our control population. However, further studies are required to evaluate the role of sCTLA-4 in RA, as well as the molecular and functional basis of the association between both *CTLA-4* gene SNPs and soluble levels of CTLA-4 in CS.

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Conflict of Interest None of the authors have any potential financial conflict of interest related to this article.

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