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ORIGINAL ARTICLE

The functional class evaluated in rheumatoid arthritis is associated with soluble TGF-β1 serum levels but not with G915C (Arg25Pro) *TGF-β1* polymorphism

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Abstract The influence of genetic factors in rheumatoid arthritis (RA) has been described, including several cytokine genes such as transforming growth factor β (TGF-β) with regulatory effects on lymphocytes, dendritic cells, macrophages, chondrocytes, and osteoblasts, which are important in the RA pathogenesis. The G915C *TGF-β1* polymorphism has been associated with soluble TGF-β1 (sTGF-β) serum levels. Thus, we studied the association of G915C (Arg25Pro) *TGF-β1* polymorphism with sTGF-β1 serum levels in RA. We enrolled 120 RA patients and 120 control subjects (CS). The G915C *TGF-β1* polymorphism was determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method, and sTGF-β1 serum levels were quantified using an ELISA kit. The genotype frequency of G915C *TGF-β1* polymorphism in RA and CS was G/G (91.7%), G/C (8.3%), C/C (0%) and G/G (85.8%), G/C (14.2%), C/C (0%), respectively, without significant differences. Moreover, the G/G *TGF-β1* genotype carriers presented the highest disability index evaluated for the Spanish HAQ-DI score ($P < 0.001$). In addition, the sTGF-β1 serum levels were higher in RA (182.2 ng/mL) than CS (160.2 ng/mL), there was not significant difference. However, we found a positive correlation between the sTGF-β1 serum levels and the functional class ($r = 0.472$, $P = 0.023$). In conclusion, the G915C (Arg25Pro) *TGF-β1* polymorphism is not associated with RA, but the sTGF-β1 serum levels are related with the functional class in RA.

Keywords Rheumatoid arthritis · Polymorphism · TGF-β1 · Functional class

Introduction

Rheumatoid arthritis (RA) is a chronic, destructive, and inflammatory disease that involve synovial joints, especially of the hands and feet [1, 2], which is characterized by

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progressive joint damage and chronic disability [3]. The characteristic in the joint is the excessive proliferation of synovial cells, antigen-presenting cells, and infiltrating leukocytes, where both T and B cells mediate the inflammatory network that contributing to the joint damage [4, 5]. There is evidence of genetic factors in RA, including several cytokine genes, which have been considered as possible candidates to influence the susceptibility of the disease [6].

Transforming growth factor β (TGF-β) has been considered an important cytokine in RA with pro- and anti-inflammatory effects [6]. Three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) have been described, which have a broad range of biological functions including wound healing, fibrosis, immune suppression, and angiogenesis [6, 7]. TGF-β1 is the most abundant isoform in mammalian [8], and it is present in synovial tissue of RA patients. In addition, it has regulatory effects on lymphocytes, dendritic cells, macrophages, chondrocytes, and osteoblasts, which are important cells in the RA pathogenesis. Moreover, the TGF-β1 has chemotactic properties with capacity to stimulate cells to produce cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) [6, 9].

The *TGF-β1* gene is located on chromosome 19q13.2. Three single-nucleotide polymorphisms within the promoter *TGF-β1* gene have been identified: C-988A, G-800A, and C-509 T. In addition, a C insertion at position +72 of the non-translated region, two polymorphisms in the signal peptide sequence at positions T869C (Leu10Pro) and G915C (Arg25Pro), and other in the precursor part of the protein C788T (Thr263Ile) have been identified [10–13]. The C-509T, T869C, and G915C polymorphisms have been show to be associated with the soluble TGF-β1 (sTGF-β1) serum levels [10]. Further, the homozygous G915G genotype (Arg25Arg) was associated with higher sTGF-β1 serum levels than heterozygous G915C genotype (Arg25Pro) [13, 14]. The G915C *TGF-β1* polymorphism has been studied in several diseases such as myocardial infarction and stroke [11], systemic lupus erytematosus [12], non-syndromic cleft lip, alveolus, and palate (CLPs) [13], juvenile idiopathic arthritis [14], ankylosing spondylitis [15], human longevity [16], and primary Sjögren's syndrome [17]. Here, we studied the association of G915C *TGF-β1* polymorphism with sTGF-β1 serum level in RA patients.

Methods

Patients

We enrolled 120 RA patients from the Hospital Civil "Fray Antonio Alcalde" Rheumatology Service, Guadalajara, Jalisco, México, who fulfilled the 1987 classification criteria of the American College of Rheumatology [18]. Spanish HAQ-DI (Spanish version of the health assessment questionnaire disability index) [19] and Functional Class (grades I–IV) [20] scores were applied in RA patients in order to measure the functional ability. The disease activity was determined by DAS28 (disease activity score using 28 joint counts) score [21]. As a control group, we included 120 control subjects (CS) residents from Guadalajara, Jalisco, Mexico.

Ethical consideration

Informed written consent was obtained from all subjects before enrollment to the study. The investigation was performed according to the ethical guidelines of the 2008 Declaration of Helsinki and was approved by the ethical committee of the Hospital Civil "Fray Antonio Alcalde", Guadalajara, Jalisco, Mexico.

Laboratory assessment

White blood cells (WBC) and platelets count (PLT) were determined in all subjects included in the study (CELL-DYN 3700; Abbott Diagnostics, North Chicago, Illinois). Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen, and rheumatoid factor (RF) were assayed in all participants (CELL-DYN 3500R, Abbott Diagnostics).

Genotyping of the G915C (Arg25Pro) *TGF-β1* polymorphism

Genomic DNA (gDNA) was extracted from peripheral blood leukocytes according to the Miller method [22]. The G915C (Arg25Pro) *TGF-β1* polymorphism was screened by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method, using the following primers 5′-TTCCCTCGAGGCCCTCCTA-3′ (Forward) and 5′-GCCGCAGCTTGGACAGGATC-3′ (Reverse) [23]. The PCR system was carried out in a final volume of 25 μL containing 1 μg of gDNA, 3 μM of each primer, 2 U/μL of *Taq* DNA polymerase (Invitrogen™ life technologies), 2.5 μL of supplied 10X buffer, 2.5 mM $MgCl_2$, 1.4 M of Betaine + 6% DMSO (SIGMA Life Science), and 2.5 mM of each dNTP (Invitrogen™ life technologies) under the following conditions: initial denaturation at 96°C for 10 min, followed by 35 cycles of 96°C for 75 s, 62°C for 75 s, and 73°C for 75 s, with an ending extension at 73°C for 5 min. The PCR product resulted in a 294-bp amplified fragment analyzed on a 6% polyacrylamide gel electrophoresis, and silver staining (Native-PAGE). Allele identification was done using a

25-bp molecular weight standard (Invitrogen, Carlsbad, CA).

Restriction pattern of G915C (Arg25Pro) *TGF-β1* polymorphism

The G to C transition was identified by digestion with 3 U of *Bgl I* restriction enzyme (New England BioLabs) for 3 h in a heat bath at 37°C. The digested products were then electrophoresed on a 6% polyacrylamide gel electrophoresis, and silver staining (Native-PAGE). Digestion fragments of 131, 103, and 60 bp represent the wild-type genotype (G/G), fragments of 163, 131, 103, and 60 bp represent the heterozygote (G/C), and 163 and 131 bp represent the polymorphic genotype (C/C).

sTGF-β1 assay

sTGF-β1 levels were determined in serum samples from RA patients and CS using an ELISA kit (TGFβ1 Human, Biotrak^{TM} Easy ELISA, Amersham Biosciences, England). The assay sensitivity was 0.1 ng/mL. The sTGF-β1 serum levels were calculated from a standard curve using the corresponding recombinant human TGF-β1.

Statistical analysis

Hardy–Weinberg equilibrium and genotype and allele frequencies were tested using Chi-square test (χ^2) (STATA v.9.2). A Student *t*-test was used for means comparison in both groups. Mann–Whitney *U* test was used for non-parametric distribution data. Differences in sTGF-β1 levels and other biological assessments were evaluated by Pearson's correlation (rho). In order to test the relationship between sTGF-β1 levels and Spanish HAQ-DI, Functional Class, and DAS28 indexes, a Spearman's correlation (r_s) was performed. Analysis was performed using SPSS 11.5, STATGRAPHICS 4.0 and GraphPad Prism statistical program. In each test, a *P* value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics of RA patients

Clinical and demographic characteristics are described in Table 1. A total of 120 RA patients were included, 113 were female and 7 male with a mean of age of 46.7 years (range 22–84). The disease duration mean since diagnosis was 9.86 years. These patients had clinical activity of the disease according to DAS28 index (mean of 5.15 score) and a disability measurement by Spanish HAQ-DI (mean of 0.91 score). The functional class grade I was the most frequent (50/120). The RA patients were treated with non-steroidal anti-inflammatory and disease modifying anti-rheumatic drugs. Respect to the biological assessment, increased levels of acute phase reactants, RF, WBC, and PLT in RA patients versus CS were observed ($P < 0.05$; data not shown).

Table 1 Baseline characteristics of RA patients and CS

Characteristics	RA ($n = 120$)	CS ($n = 120$)
Demographics		
Age, years (range)	46.7 (22–84)	38.2 (18–69)
Sex F/M	113/7	87/33
Disease duration, year (range)	9.86 (0.11–32)	ND
Clinical assessment		
Swollen joints, count 28	6.9 (0–24)	ND
Painful joints, count 28	8.13 (0–28)	ND
Patient's global assessment of disease status (0–10 VAS)	5.0 (0–10)	ND
DAS score	5.15 (1.13–8.42)	ND
Spanish HAQ-DI score	0.91 (0.0–2.83)	ND
Functional class		
I	50/120	ND
II	44/120	ND
III	23/120	ND
IV	3/120	ND
Drug treatment		
Prednisone < 8.5 mg/day	30/120	ND
DMARDs	72/120	ND
NSAIDs	85/120	ND

Values represent the mean, minimum, and maximum scores

RA rheumatoid arthritis, *CS* control subjects, *VAS* visual analog scale, *DAS28* disease activity score, using 28 joint counts, *Spanish HAQ-DI* Spanish version of the health assessment questionnaire disability index, *DMARDs* disease modifying anti-rheumatic drugs, *NSAIDs* non steroidal anti-inflammatory drugs, *F* female, *M* male, *ND* no determined

Genotype and allele frequencies of G915C (Arg25Pro) *TGF-β1* polymorphism

Our population was in Hardy–Weinberg equilibrium ($X^2 = 0.69$, $P = 0.40$). The genotype and allele frequencies of the G915C (Arg25Pro) *TGF-β1* polymorphism showed no significant differences between RA patients and CS (Table 2). However, the G/G genotype was the most frequent in RA, and the homozygous C/C genotype was not observed in both studied groups. The RA patients were divided according to the genotypes obtained (G/G $n = 110$; G/C $n = 10$). We observed the association between G/G *TGF-β1* genotype carriers and the highest disability index evaluated for the Spanish HAQ-DI score

Table 2 Genotype and allele frequencies of G915C (Arg25Pro) *TGF-β1* polymorphism in RA patients and CS

	RA ($n = 120$) %(n)	CS ($n = 120$) %(n)	P value
Genotype			
G/G	91.7 (110)	85.8 (103)	
G/C	8.3 (10)	14.2 (17)	0.15 (NS)
C/C	0 (0)	0 (0)	
Allele			
G	95.8 (230)	92.9 (223)	0.53 (NS)
C	4.2 (10)	7.1 (17)	

RA rheumatoid arthritis, *CS* control subjects, *NS* not significant

($P < 0.001$; Table 3). The other clinical and biological characteristics showed no significant differences.

G915C (Arg25Pro) *TGF-β1* polymorphism and sTGF-β1 serum levels

The sTGF-β1 serum concentrations in RA patients and CS were measured and examined for their association with the G915C (Arg25Pro) *TGF-β1* polymorphism. Although the sTGF-β1 serum levels were higher in RA patients than CS, the difference was not significant (182.2 ± 18.6 ng/mL vs. 160.2 ± 11.0 ng/mL, respectively; $P = 0.330$) (Fig. 1a). Nevertheless, the analysis of the sTGF-β1 serum levels according to G/G and G/C genotypes in RA patients not revealed significant differences (Fig. 1b).

Correlation of the sTGF-β1 serum levels with clinical characteristics in RA patients

The sTGF-β1 serum levels were correlated with DAS28, Spanish HAQ-DI, and functional class indexes. The DAS28 and Spanish HAQ-DI indexes showed a negative correlation with the sTGF-β1 serum levels ($r = -0.120$, $P = 0.575$; $r = -0.008$, $P = 0.968$; respectively) (data not shown). However, we found a positive correlation between the functional class and the sTGF-β1 serum levels ($r = 0.472$, $P = 0.023$) (Fig. 2).

Discussion

In this study, we examined the G915C (Arg25Pro) *TGF-β1* polymorphism and their relationship with sTGF-β1 levels in RA. The G915C (Arg25Pro) *TGF-β1* polymorphism was not associated with susceptibility to the disease. However, the sTGF-β1 serum levels were associated with the grade of functional class (I–IV) in RA patients. Thus, this is the first report to show that sTGF-β1 serum levels may be involved with the clinical activity in RA patients.

Previous studies have shown that TGF-β1 is produced in large amounts in synovial fluids of RA patients. The synovial cells are also able to produce TGF-β1 in vitro and in vivo. This cytokine has been associated with remission of arthritis by the anti-inflammatory effects [24]. In our study, higher sTGF-β1 levels in RA than CS were found, without significant difference. However, a positive correlation between sTGF-β1 levels and the grade of functional class (I–IV) was observed in RA patients ($r = 0.472$, $P = 0.023$). This data indicate that the increased sTGF-β1 levels in RA are in relationship with the functional class and the clinical activity. This finding can be explained because TGF-β1 has been considered an important modulator of the immune response in RA and might have pro- and anti-inflammatory effects. In addition, TGF-β1 has chemotactic properties and may stimulate cells to produce IL-1, IL-6, and TNFα at inflammation sites [6]. Moreover, TGF-β1 also has adverse effects on synovial inflammation

Table 3 Demographic and clinical characteristics in RA patients according to G915C (Arg25Pro) *TGF-β1* polymorphism

Characteristics	Genotype		
	G/G ($n = 110$)	G/C ($n = 10$)	P value
Demographics			
Age, years (range)	42.6 (18–84)	41.5 (18–67)	0.68
Disease duration, year (range)	10.0 (0.11–32)	8.2 (0.83–17)	0.39
Clinical assessment			
Swollen joints, count 28	7.1 (0–24)	4.3 (0–15)	0.14
Painful joints, count 28	8.2 (0–28)	7 (0–28)	0.70
Patient′s global assessment of disease status (0–10, VAS)	5.0 (0–10)	4.3 (1–7)	0.29
DAS score	5.2 (1.13–8.42)	4.64 (2.12–6.48)	0.26
Spanish HAQ-DI score	0.91 (0–2.83)	0.33 (0–1.47)	**<0.001**

Values represent the mean and minimum and maximum scores. $P > 0.05$

VAS visual analog scale, *DAS28* disease activity score using 28 joint counts, *Spanish HAQ-DI* Spanish version of the health assessment questionnaire disability index

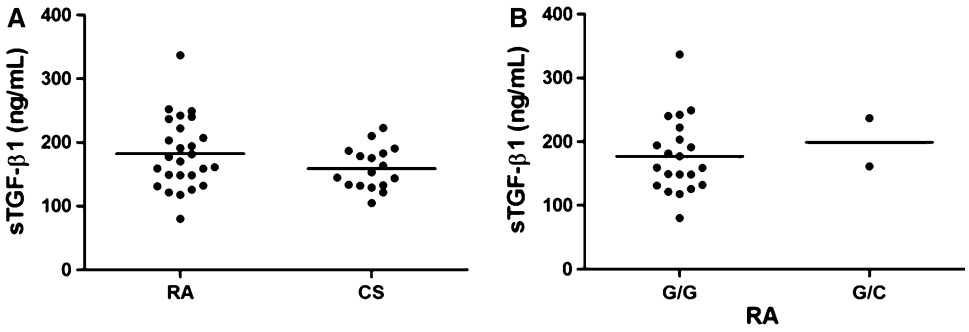

Fig. 1 sTGF-β1 serum levels in RA patients and CS. **a** sTGF-β1 serum levels in RA patients and CS. **b** sTGF-β1 serum levels in RA patients according to G/G and G/C genotypes

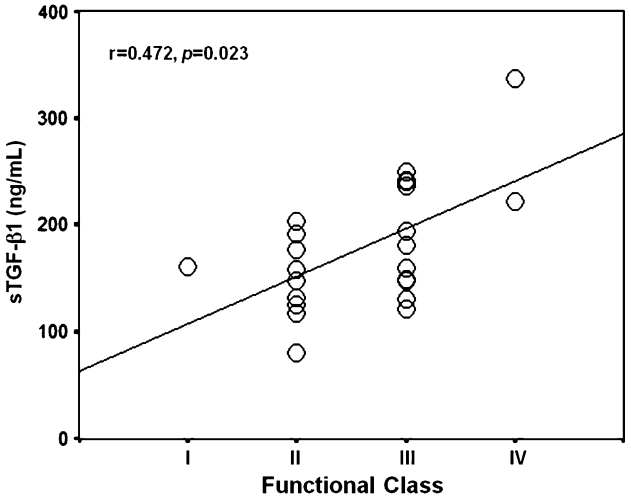

Fig. 2 Correlation between the serum levels sTGF-β1 and the functional class evaluated in RA patients

because it induces the expression of vascular endothelial growth factor (VEGF) in synovial fibroblast [14].

Our results demonstrate lack of association of G915C (Arg25Pro) *TGF-β1* polymorphism with RA. Here, we showed that the frequency of the G allele (95.8%) in RA was higher than CS (92.9%) without significant differences, whereas the C allele (7.1%) was more common in CS versus RA (4.2%). The G/G genotype in RA and CS was the most frequent (91.7% vs. 85.8%, respectively). In contrast, the frequency of G/C genotype was higher in CS (14.2%) than RA (8.3%). In both studied groups, the homozygous C/C was not observed. These results are in agreement with the study of Kim et al. [25], who reported lack of association with G915C (Arg25Pro) *TGF-β1* polymorphism in Korean RA population and also the C/C genotype was not identified but the G/C genotype was observed in a low frequency (0.7%) in both studied groups. However, in the same study, another *TGF-β1* polymorphism (−509T) was associated with the progression of radiographic severity in RA patients [25]. Oen et al. [14] demonstrated association between the G/G genotype of the G915C (Arg25Pro) *TGF-β1* polymorphism with decreased risk to radiographic damage in juvenile rheumatoid arthritis (JRA) [14], however, Cinek et al. [26], not observed significant differences with G915C (Arg25Pro) *TGF-β1* polymorphism in JRA [26].

When we compared the G915C (Arg25Pro) *TGF-β1* polymorphism with the sTGF-β1 serum levels, the clinical and demographic characteristics in RA, only the G/G *TGF-β1* genotype carriers presented the highest disability index evaluated for the Spanish HAQ-DI score, this data suggest that the *TGF-β1* polymorphism could be involved in the RA course. Previously, the 915G allele has been associated with increased production of TGFβ protein both in vitro and in vivo studies. A possible explanation of this finding has been highlighted because the polymorphism at codon 25 is located within the signal peptide that is cleaved from the TGF-β1 precursor until codon 29. It is possible that the change of Arg by Pro could play a role in the production of the mature TGF-β1. In addition, the signal peptide allows exporting the cytokine precursor across the membrane of the endoplasmic reticulum it could suggest that the polymorphism affect this process [16].

In conclusion, the G915C (Arg25Pro) *TGF-β1* polymorphism is not associated with RA, but the sTGF-β1 serum levels are related with the functional class in RA patients.

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Conflict of interest The authors declare that they do not have any conflict of interest.

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