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Association of CD28 IVS3 +17T/C polymorphism with soluble CD28 in rheumatoid arthritis

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Abstract. *Objective:* Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology in which inflammatory pathology involves T cell activation and the CD28 costimulatory molecule involved in T cell presentation. The gene includes the CD28 IVS3 +17T/C polymorphism that could be associated with susceptibility to RA whereas the soluble concentrations of CD28 (sCD28) could be related to clinical activity.

Methods: We investigated the CD28 IVS3 +17T/C polymorphism in 200 RA patients and 200 healthy subjects (HS). Furthermore, we quantified the sCD28 concentrations in 77 samples of each group. We applied indexes focused to determine the activity and disability (DAS28 and Spanish HAQ-DI, respectively) in RA patients.

Results: RA patients had significantly higher frequencies of the CD28 T allele compared to HS ($p = 0.032$ OR $= 1.59$, C.I. 1.02–2.49). In addition, the IVS3 +17 T/T genotype frequency was also increased in RA vs. HS ($p = 0.026$). The RA patients showed higher sCD28 serum levels than HS ($p = 0.001$). Carriers of the T/T genotype in RA patients showed higher sCD28 levels than C/C carriers ($p = 0.047$). In addition, a correlation between sCD28 and Spanish HAQ-DI (correlation, 0.272; $p = 0.016$), was found.

Conclusion: The T allele in CD28 IVS3 +17T/C polymorphism is associated with a susceptibility to RA in Western Mexico. In addition, increased sCD28 levels are related to T/T genotype in RA patients.

Keywords: CD28, IVS3 +17T/C, polymorphism, rheumatoid arthritis, sCD28

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by progressive sinovitis and the formation of *pannus*, which can lead to articular damage and the loss of function in the diarthrodial joints. This process involves T cell activation and hyperactivity which

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have been related to an irregular function of the costimulatory molecules in autoimmune diseases [1].

CD28 (mCD28) in membranes is an important costimulator of T lymphocytes, their principal events are increasing the response and promotion of T cells survival through positive signalization [2]. Moreover, soluble CD28 (sCD28) could regulate T cell proliferation and contribute to the loss of their own tolerance [3], in competition with their ligands B7.1 (CD80) and B7.2 (CD86).

The IVS3 +17T/C single nucleotide polymorphism (SNP) of *CD28* gene, with a T/C substitution at position +17 in the third intron, is located in the $2q33$ region [4]. This polymorphism has been related with Behçet disease [5], but has not been considered in other inflammatory diseases like RA. Based on the importance of this molecule our purpose was to determine if *CD28* IVS3 +17T/C polymorphism and sCD28 could be related to susceptibility and clinical activity to RA in Western Mexico.

2. Subjects, materials and methods

This study included 200 RA patients (190 female, 10 male; mean age 48 $\pm$ 14) classified according to the American College of Rheumatology criteria [6] from the Rheumatology Departments of the O.P.D. Hospital Civil Fray Antonio Alcalde and Hospital Valentín Gómez Farias, Jalisco, Mexico. As a control group, we recruited 200 healthy subjects (HS) (123 female, 77 male; mean age 35 $\pm$ 11.8). The RA patients and HS were born in Western Mexico with similar genetic background and a family history of Mexican ancestors, at least back to the third generation. Disease activity and disability in RA patients were evaluated through the Disease Activity Score using 28 joint counts (DAS28) and Health Assessment Questionnaire (Spanish HAQ-DI), respectively. The internal Committee of Ethics of both Institutions mentioned above, approved the present study in compliance with the Helsinki declaration (CE3/CI-03 and No. 1934/07). Informed consent was obtained from both study groups.

2.1. *Polymerase Chain Reaction* (*PCR*) – *Restriction fragment length polymorphism* (*RFLP*) *analysis of CD28 IVS3 +17T/C*

Genomic DNA was isolated from peripheral blood leucocytes using standard procedures. In order to amplify the 147 bp region that includes the *CD28* IVS3 +17T/C polymorphism, we used the following primers for the PCR reaction: forward 5'TTTTC TGGGTAAGAGAAGCAGCGC-3' and reverse 5'-GA ACCTACTCAAGCATGGGG-3'. Each of the PCR reactions included 5 μL of DNA, 3 μM of each primer, 2.5 μL of 1X reaction buffer (Invitrogen®), 1.5 mM magnesium chloride (Invitrogen®), 2.5 mM dNTPs (Invitrogen®), and 0.125 μL of *Taq* DNA polymerase (Invitrogen®) in a total volume of 18 μL.

The *CD28* gene amplification conditions were as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 2 min.

2.2. *CD28 IVS3+17T/C restriction pattern polymorphism*

The T to C transition was identified by digestion with 5 U of *AfeI* restriction enzyme (New England BioLabs®) for 3 h at 37°C. Digested products were electrophoresed on a 3% agarose gel (Invitrogen Life Technologies®) and then stained with ethidium bromide. Digestion fragments of 125 and 22 bp represent the wild type genotype (T/T); fragments of 147, 125 and 22 bp represent the heterozygote (T/C) whereas 147 bp represents the polymorphic genotype (C/C). To confirm this technique, random samples were taken of each genotype and sequenced using an ABIPRISM 310 Sequencer (Applied Biosystem, Forter City, CA®).

2.3. *Assay of serum sCD28*

Serum concentrations of sCD28 from 77 RA patient and 77 HS were measured by enzyme-linked immunosorbent assay (ELISA) using reagent kits for human sCD28 (Bender Medsystems Diagnostics, Human sCD28BMS290®). The assay sensitivity was $<$ 0.18 ng/mL. The sCD28 production was calculated from a standard curve of the corresponding recombinant human sCD28

2.4. *Statistical analysis*

Genotype frequencies in this study were tested for Hardy-Weinberg equilibrium using a chi-square test. Differences between genotype and allele frequencies of both groups were evaluated by chi-square analysis and Fisher's Exact test. The Mann-Whitney test was used to compare mean sCD28 concentrations. The Spearman correlation was used to compare sCD28 with DAS28 and with Spanish HAQ-DI. All p values reported for

Table 1
Demographic and clinical characteristics of RA patients

Characteristics	RA ($n = 200$)
Demographic	
Age, years (range)	48 (22–86)
Sex (F/M)	190/10
ACR criteria	
FR positive	48.3%
Arthritis at least on 3 joints	56.1%
Symmetric arthritis	53.5%
Arthritis on hands	68.7%
Morning stiffness	80.2%
Rheumatic nodules	27.3%
Clinical	
DAS28 score	4.86 (1.13–8.42)
Painful joints, count 28	6.69 (0-28)
Swollen joints count 28	5.34 (0–24)
Patient's global assessment of disease status (0–10 VAS)	5.03 (0–10)
Spanish HAQ-DI score	0.76 (0.00–2.83)
Disease duration, years (range)	11.16 (0.11–52)
Drug treatment	
Prednisona $<$ 8.5 mg/day	35/200
DMARDs:	153/200
Methotrexate	114/200
Chloroquine	99/200
Azulfidine	46/200
Azatioprine	7/200
NSAIDs	150/200

Values represent the mean, minimum and maximum range; some of them represent percentage;
RA rheumatoid arthritis, F female, M male, FR rheumatoid factor, *VAS* visual analogue scale, *DAS28* Disease Activity Score using 28 joint counts, *Spanish HAQ-DI* Spanish version of Health Assessment Questionnaire Disability Index, *DMARDs* Disease Modifying Anti-Rheumatic Drugs, NSAIDs non steroideal anti-inflammatory drugs.

comparisons and correlations are two-sided and considered significant when $p < 0.05$. We used the SPSS (SPSS Inc.) v15.0 statistical package for all statistical analysis.

3. Results

3.1. Patient characteristics

The main demographic and clinical characteristics of RA patients are summarized in Table 1. The patients had a moderate clinical activity and mild disability defined by DAS-28 (4.86 score) and HAQ-DI (0.76 score) indexes, respectively. The average disease duration was 11 years and they were treated with disease modifying antirheumatic drugs (DMARDs) and non-steroidal anti-inflammatory drugs (NSAIDs).

3.2. Genotype and allele frequencies of the CD28 IVS3 +17T/C polymorphism

Our population was in Hardy-Weinberg equilibrium for the CD28 IVS3 +17T/C polymorphism ($p > 0.05$). The distribution of genotypes and alleles frequencies in RA and HS are presented in Table 2. The results showed a significant difference between the frequency of CD28 IVS3 +17T/C genotypes in RA and HS ($p = 0.026$). The RA patients had significantly higher frequencies of the CD28 T allele compared to HS (90% vs. 85%, $p = 0.032$ OR $= 1.59$, C.I. 1.02–2.49), whereas the C allele frequency was significantly lower in RA than HS (10% vs. 15%).

3.3. Serum soluble CD28 concentrations

The soluble concentrations of CD28 were significantly higher in RA (1.2 ± 1 ng/mL) than in HS (0.85 ± 0.47 ng/mL) ($p = 0.0017$) (Fig. 1a). We analyzed the sCD28 levels in relationship to the CD28 IVS3 +17T/C polymorphism in RA patients, when was observed higher levels of sCD28 in the T/T genotype (1.5 ± 1.3 ng/mL) than in the T/C carriers (0.90 ± 0.31 ng/mL) ($p = 0.0338$) (Fig. 1b). No significant differences between sCD28 levels and each genotype identified in HS, were observed (data not shown).

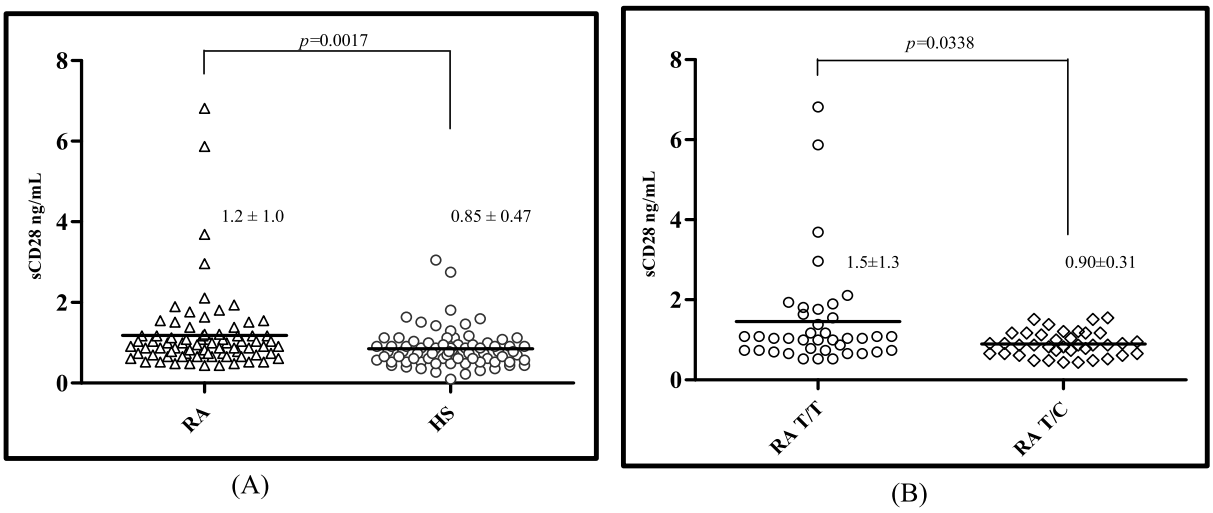

Fig. 1. Soluble CD28 levels in serum of RA patients and HS. sCD28 was detected by ELISA. Data were analyzed with a Mann-Whitney test.

Table 2
Genotype and allele frequency of IVS3 +17T/C polymorphism in RA and HS

	RA % (n)	HS % (n)	P	OR (95% CI)
Genotype				
T/T	80 (160)	72.5 (145)	*0.026	
T/C	20 (40)	25 (50)		
C/C	0 (0)	2.5 (5)		
Allele				
T	90 (360)	85 (340)	‡0.032	1.59 (1.02–2.49)
C	10 (40)	15 (60)		

*Genotype analysis with Fisher's Exact test, $p = 0.026$;
‡Allele analysis with χ^2 (1) = 4.57 $p = 0.032$;
RA rheumatoid arthritis, HS Healthy subjects, n number, OR odds ratio, CI confidence interval.

The sCD28 concentrations in DAS28 index not showed a significant correlation ($r = 0.207$; $p = 0.071$). The sCD28 concentrations exhibited a positive and significant correlation with the Spanish HAQ-DI score ($r = 0.272$; $p = 0.016$).

4. Discussion

The costimulation is a very important process in development of RA immunopathology. The CD28 molecule is one of the principal costimulatory molecules for T cell activation. The main effects of CD28 are to increase the response and promote T cell survival [2].

In spite of the close relationship between the costimulation and RA physiopathology, CD28 IVS3 +17T/C SNP has not been associated with this disease. Our results in the Western Mexico showed that T/T genotype was higher in RA patients (80%) than HS (72.5%). In addition, the T allele frequency was higher in RA vs HS (90% vs. 85%). This findings suggest that T allele is associated with susceptibility to RA from the Western Mexico population while the C/C genotype was absent in RA group. Until now, this polymorphism has not been studied in another RA population. However, the CD28 IVS3 +17T/C polymorphism has been associated with Behçet disease [5] but not with systemic lupus erythematosus (SLE) [4].

In agreement with sCD28 studies in SLE, Sjögren's syndrome and systemic sclerosis [3], our study showed higher sCD28 concentrations in RA than HS. The sCD28 levels can be produced by membrane shedding or mRNA alternative splicing [3,7,10]. High levels of sCD28 in RA patients could suggest a regulatory mechanism to compensate the activation in T cell. It process could reflect an inadequate T cell activation, and it could be contributing to the loss of tolerance, influencing the autoimmunity and increasing the severity in autoimmune diseases [3,7,11]. In addition, we compared sCD28 levels according to each IVS3 +17T/C genotypes in RA patients where higher sCD28 levels in T/T carriers compared to the T/C genotype, was found. These data advocate the influence of the T allele on the soluble levels. Besides, we found a significant correlation between sCD28 levels and Spanish HAQ-DI, it finding suggest that higher levels of sCD28 are in relationship with disability function in RA patients. We did not find a significant correlation with DAS28, probably because in the present study were included RA patients with different activity of the disease.

The sCD28 levels could act like an inhibitory molecule, preventing the interaction between CD28 on

the surface membrane and their receptors. This suggests that sCD28 affinity for CD80/86 is lower than CD28 on the membrane surface. In addition, the CD28 signal depends on the ligands affinity. Magistrelli et al., reported that resting T cells can express mCD28 and sCD28, and both can share positive as well as negative mechanisms to regulate T cell activation [10].

In conclusion, the T allele in CD28 IVS3 +17T/C polymorphism is associated with a susceptibility to RA in Western Mexico. Furthermore, increased sCD28 levels are related to T/T genotype in RA patients.

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