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ORIGINAL ARTICLE

Tumor necrosis factor receptor 2 M196R polymorphism in rheumatoid arthritis and osteoarthritis: relationship with sTNFR2 levels and clinical features

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Abstract We investigate the clinical association of tumor necrosis factor receptor 2 (*TNFR2*) M196R polymorphism with rheumatoid arthritis (RA) and knee osteoarthritis (OA). Acute phase reactants, lipid profile, sTNFR2 levels, disease activity–disability indexes, and *TNFR2* M196R polymorphism were analyzed in 50 RA, 50 knee OA patients, and 120 healthy subjects (HS). The M/M genotype frequency was 0.74 (RA), 0.80 (OA), and 0.64 (HS). The M/R genotype frequency was RA (0.26), OA (0.20), and HS (0.29). The R/R genotype was observed only in HS (0.07). The M allele was associated with OA ($P = 0.0137$, OR = 2.43). Total cholesterol, triglyceride levels, apolipoprotein A-I and B showed significant differences ($P < 0.05$). The highest sTNFR2 levels were observed in RA and OA ($P = 0.001$), however M/M and M/R carriers do not correlate with sTNFR2 production. Our findings suggest an association of the M allele with knee OA. In addition, high sTNFR2 levels in RA and OA were found.

Keywords Tumor necrosis factor receptor 2 · Polymorphism · Rheumatoid arthritis · Osteoarthritis

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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease with striking individual, social, and economic impact. The RA inflammatory process involves cellular and molecular mechanisms characterized by progressive joint damage mediated by interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) that stimulate the expression of adhesion molecules on endothelial cells and recruitment of monocytes and neutrophils into the joint space. TNFα by itself is a potent inducer of metalloproteinase (MMPs) production by synoviocytes therefore participates actively in joint damage process [1, 2].

Osteoarthritis (OA) is the most common form of arthritis, and the social impact includes cost-effective medical care and work disability. The OA pathophysiology involves proteinases (MMPs, aggrecanases,

cathepsins, and elastases) and inflammatory factors induced by IL-1 and TNFα [3, 4]. In both diseases, TNFα plays a pivotal role and its effects are regulated by two receptors: the 55 kDa TNF receptor 1 (TNFR1, CD120a, TNFRSF1a) and the 75 kDa TNF receptor 2 (TNFR2, CD120b, TNFRSF1b) [5]. The *TNFR2* gene is located on the chromosome 1p36.2 which is composed by ten exons and nine introns. On exon 6, position 196, a single nucleotide polymorphism (T/G) resulting in an amino acid modification in the fourth extracellular cysteine rich domain (CDR4) from methionine (M) to arginine (R) has been reported. The functional importance of this amino acid substitution has been postulated that could affect in three processes: (1) exon 6 encodes for proteolytic cleavage site of TNFR2 resulting in the generation of soluble form, (2) the optimal TNF binding is affected, and (3) the *TNFR2* polymorphism affects TNFα induced apoptosis and impaired NFκB signaling [6, 7].

An association of *TNFR2* M196R polymorphism with the pathogenesis of systemic lupus erythematosus (SLE) in Japanese patients but not in Spanish and UK population has been reported [8, 9]. High levels of sTNFR2 have been found in serum of patients with SLE, RA, osteosarcoma, and coronary artery disease [10–13]. Based on this knowledge, we investigated the association of the *TNFR2* M196R polymorphism with sTNFR2 levels in RA and OA patients.

Materials and methods

Patients and healthy subjects

Fifty RA and 50 OA patients were enrolled from the Hospital Civil "Fray Antonio Alcalde", Rheumatology Service, from December 2001 to April 2003. All patients fulfilled the 1987 and 1986 classification criteria for RA and knee OA, respectively, according to American College of Rheumatology. Spanish HAQ-DI, Spanish-AIMS and DAS28 activity and disability indexes were applied to RA patients at the beginning of the study [14–16]. WOMAC and Lequesne disability indexes were applied to OA patients [17, 18]. As a control group, 120 healthy subjects (HS) who had the same ethnic and geographical background, residents from Guadalajara were included. All HS participants were selected according to their age and gender.

Ethical consideration

Informed written consent was obtained from all subjects before enrollment to the study according to the ethical guidelines of 2000 Declaration of Helsinki.

Laboratory assessment

Erythrocyte sedimentation rate (ESR), white blood cell count (WBC), red blood cell count (RBC), platelet count (PLT) (CELL-DYN 3700, Abbott Diagnostics), C-reactive protein (CRP), and rheumatoid factor (RF) were determined in all the participants. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), very low-density lipoprotein cholesterol (VLDL-c) were assayed according to SYNCHRON CLINICAL SYSTEM LX20 of FALCON methods. Apolipoprotein A-I (apoA-I) and apolipoprotein B (apoB) levels were measured according to manufacturer assay (IMMAGE™ Immunochemistry, Beckman Coulter System 4700).

Genotype analysis of *TNFR2* M196R polymorphism

Genomic DNA was extracted from leukocytes obtained from whole blood samples, according to the Miller method [19]. Amplification was done by PCR in a Thermal Cycler (Techne, TC-312) using the following primers, 5′ ACT CTC CTA TCC TGC CTG CT 3′ (forward) and 5′ TTC TGG AGT TGG CTG CGT GT 3′ (reverse). The PCR was carried out in a final volume of 50 μl containing 1 μg of gDNA, 3 μM of each primer, 1.25 U/μl *Taq* DNA polymerase (Invitrogen™ life technologies), 5 μl of supplied 10× buffer enzyme, 1 mM $MgCl_2$, and 0.1 mM of each dNTP (Invitrogen™ life technologies). The PCR was performed by initial denaturation at 94°C for 3 min, followed by 35 amplification cycles at 94°C during 30 s for denaturation, 57°C during 30 s for annealing and 72°C during 30 s for extension, and finally, 72°C during 1 min for ending extension. The PCR product resulted in a 242-bp amplified fragment analyzed on a 2% agarose (Invitrogen™ life technologies) gel stained with ethidium bromide. The amplified fragment was incubated with 3 U of *Nla*III restriction enzyme (New England BioLabs) for 2 h in a heat bath at 37°C. Restriction fragments were analyzed on a 4% agarose gel (Invitrogen™ life technologies) stained with ethidium bromide.

Genotype analysis

The wild genotype (M/M) corresponds to 133 and 109 bp fragments; heterozygote genotype (M/R) is represented by 242, 133, and 109 bp fragments; and homozygote genotype (R/R) corresponds to 242 bp fragment. Each genotype was sequenced using an

ABIPRISM 310 Sequencer (Applied Biosystems) in order to confirm the results.

sTNFR2 assay

sTNFR2 production was measured using serum samples from RA, OA patients and HS by ELISA (R&D Systems, Minneapolis, MN, USA). The sensitivity of the assay was 0.6 pg/ml. sTNFR2 levels were calculated from a standard curve using the corresponding recombinant human TNFR2.

Statistical analysis

Genetic and allelic frequency differences between groups were tested using Chi-square test (χ^2), odds ratio (OR) with 95% confidence interval (95% CI). A student t test was used for two group means comparison. Mann–Whitney U test was used for nonparametric distribution data. Differences in sTNFR2 levels and other biological assessments were evaluated by Pearson's correlation (rho). In order to test the relationship between sTNFR2 levels and Spanish HAQ-DI, Spanish-AIMS, DAS28, WOMAC and Lequesne indexes, Spearman's correlation (r_s) was performed. Probability values less than 0.05 were considered significant. Analysis was performed using SPSS 10.0 software.

Results

TNFR2 M196R polymorphism

Fifty RA, 50 knee OA patients, and 120 HS were genotyped for *TNFR2* M196R polymorphism. Our population was in Hardy–Weinberg equilibrium. The M/M genotype was the most frequent in patients and HS (0.74, 0.80, 0.64; RA, OA, and HS, respectively). No difference between RA, OA, and HS was found in genotype frequency, moreover M allele was more frequent in OA group ($P = 0.0137$, OR = 2.43, 95% CI = 1.13–5.36) (Table 1).

Baseline characteristics of patients

The baseline characteristics of the patients are shown in Table 2. The RA patients had 9 years diagnosed as RA and follow-up most of them with NSAIDs and DMARDs; these patients were active as indicated by DAS28 score (6.23 score). The OA group had 5 years diagnosed with evident clinical progression and 44 of them had used NSAIDs; Lequesne index demonstrates a marked muscle-skeletal disability (11.91 score). The RA and OA patients were divided into genotype group M/M ($n = 37$, RA; $n = 40$, OA) and genotype group M/R ($n = 13$, RA; $n = 10$, OA). In this case, all baseline characteristics were similar between M/M or M/R carriers (Table 2).

Biologic assessment

The RA patients demonstrate higher levels of RF, ESR, and CRP; lipid profile shows lower levels in RA with respect to OA and HS (Table 3). Despite that, no differences between M/M or M/R genotype carriers within RA or OA group were found.

TNFR2 quantification

Serum levels of sTNFR2 were measured in samples of RA and OA patients, and HS. Elevated sTNFR2 levels were found in RA patients ($P = 0.0001$ vs. OA and HS). Likewise, OA patients presented higher levels of sTNFR2 than in HS ($P = 0.023$) (Fig. 1a). Nevertheless, subjects who were M/M and M/R carriers do not demonstrate significant differences in sTNFR2 levels (Fig. 1b).

Discussion

The *TNFR2* M196R polymorphism has been associated with autoimmune disorders, including SLE in Japanese but not in Spanish or UK populations [8, 9, 20]. This study provides evidence of lack of association of

Table 1 Genotype and allele frequency of *TNFR2* M196R polymorphism in RA, OA, and HS

	RA ($n = 50$)	OA ($n = 50$)	HS ($n = 120$)	P value
Genotype frequency				
M/M	0.74 (37)	0.80 (40)	0.64 (77)	NS
M/R	0.26 (13)	0.20 (10)	0.29 (35)	
R/R	0 (0)	0 (0)	0.07 (8)	
Allele frequency				
M	0.87 (87)	0.90* (90)	0.79 (189)	$P = 0.0137$, OR 95% CI = 1.13
R	0.13 (13)	0.10 (10)	0.21 (51)	

RA rheumatoid arthritis, *OA* osteoarthritis, *HS* healthy subjects

*$P = 0.0137$ OA versus HS

Table 2 Baseline characteristics in RA, and OA patients M/M, and M/R genotype carriers to *TNFR2* M196R polymorphism

	RA				OA			
	All RA patients ($n = 50$)	M/M genotype ($n = 37$)	M/R genotype ($n = 13$)	*P*	All OA patients ($n = 50$)	M/M genotype ($n = 40$)	M/R genotype ($n = 10$)	*P*
Demographics								
Age, years	44 (22–71)	45 (22–71)	41(29–65)	–	55 (31–86)	55 (31–86)	55 (32–79)	–
Disease status								
Disease duration, years	9 (0.2–30)	10 (0.2–30)	8 (1–20)	–	5 (0.5–20)	5 (1–20)	3 (0.5–8)	–
Drug treatment								
Prednisone <8.5 mg/day	11/50	9	2	–	–	–	–	–
DMARDs	29/50	20	9	–	–	–	–	–
NSAIDs	44/50	33	11	–	42	34	8	–
Clinical assessment								
Swollen joints, 28 counts	10 (1–24)	9 (2–24)	10 (1–24)	NS	–	–	–	–
Tender joints, 28 counts	10 (0–28)	13 (1–28)	14 (1–28)	NS	–	–	–	–
Morning stiffness, min	76.70 (5–480)	78 (5–480)	71 (15–360)	NS	–	–	–	–
Patient's global assessment of disease status (0–10, VAS)	6.76 (1–10)	6 (1–10)	7 (5–10)	NS	6 (0–10)	6 (0–10)	5 (0–8)	NS
DAS28 score	6.23 (3.58–8.15)	6.30 (3.58–8.04)	6.0 (4.69–8.15)	NS	–	–	–	–
Spanish HAQ-DI score	1.20 (0.08–2.56)	1.23 (0.08–2.56)	0.93 (0.08–2.25)	NS	0.42 (0.0–1.94)	0.40 (0.0–1.83)	0.51 (0.0–1.94)	NS
Spanish-AIMS score	3.98 (2.21–5.76)	3.83 (2.21–5.41)	4.36 (2.41–5.76)	NS	–	–	–	–
WOMAC score	–	–	–	–	2.47 (0.83–3.94)	2.38 (0.83–3.94)	2.88 (1.43–3.92)	NS
Lequesne score	–	–	–	–	11.91 (3–21)	11.84 (3.0–21.0)	12.28 (7.0–17.0)	NS

Values represent the mean, minimum, and maximum scores

NS not significant, *DMARDs* disease modifying antirheumatic drugs, *NSAIDs* non steroidal anti-inflammatory drugs, *VAS* visual analogue scale, *DAS28* disease activity score using 28 joint counts, *Spanish HAQ-DI* Spanish version of the Health Assessment Questionnaire Disability Index, *Spanish-AIMS* Spanish version of the Arthritis Impact Measurement Scales, *WOMAC* Western Ontario and McMaster Universities index

Table 3 Acute phase reactants and lipid profile in RA, OA, and HS

	RA	OA	HS
RF (IU/ml)	654.41*# (20–4280)	24.26 (20–194)	20.10 (20–23)
ESR (mm/h)	41.34*# (15–65)	25.79† (7–45)	20.00 (2–50)
Serum CRP level (mg/dl)	3.02*# (0.22–18.90)	0.62 (0.10–4.01)	0.33 (0.10–1.44)
WBC (κ/μl)	7.42 (2.23–14.90)	5.81 (3.32–10.90)	6.27 (3.89–11.90)
RBC (M/μl)	4.57 (3.64–5.70)	4.75 (4.21–5.62)	4.99 (3.98–7.81)
PLT (κ/μl)	343.26 (192–638)	254.84 (137–480)	257.78 (14.30–459)
TC (mg/dl)	175.50*# (107–364)	204.56 (127–325)	199.63 (126–366)
TG (mg/dl)	110.04* (39–229)	155.18 (57–426)	137.41 (24–461)
HDL-C (mg/dl)	40.69 (16–78)	43.66 (22–86)	45.76 (21–91)
LDL-C (mg/dl)	112.11* (53.80–274.80)	131.07 (69.20–208.80)	126.74 (78–257)
VLDL-C (mg/dl)	22 (7.80–45.80)	37.90 (11.40–333)	32.18 (4.80–531)
apoA-I (mg/dl)	158.65# (50.60–363)	171.58† (83–440)	139.83 (39.20–237)
apoB (mg/dl)	106.06* (40.30–281)	134.44† (63.20–360)	98.97 (40.40–177)

Values represent the mean, minimum, and maximum scores

RF rheumatoid factor, *ESR* erythrocyte sedimentation rate, *CRP* C reactive protein, *WBC* white blood cells, *RBC* red blood cells, *PLT* platelets, *TC* total cholesterol, *TG* triglyceride, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *VLDL-C* very low-density lipoprotein cholesterol, *apoA-I* apolipoprotein A-I, *apoB* apolipoprotein B

*$P < 0.05$ versus OA, #$P < 0.05$ versus HS, †$P < 0.05$ versus HS

the *TNFR2* M196R polymorphism in Mexican RA patients. However, Barton et al. [21] reported a strong association of the R allele and the R/R genotype of *TNFR2* polymorphism with family RA in UK patients. On the other hand, Bridges et al. [22] suggest no association of this polymorphism in African Americans with a family history of RA. In addition, van der Helm-van Mil et al. [23] suggested no association of the *TNFR2* polymorphism with disease severity in Dutch RA patients even though they compared extreme phenotypes: patients with complete remission and patients with severe progression. The divergence of results of this study and the results previously reported in RA patients demonstrate the inter-population genetic variability.

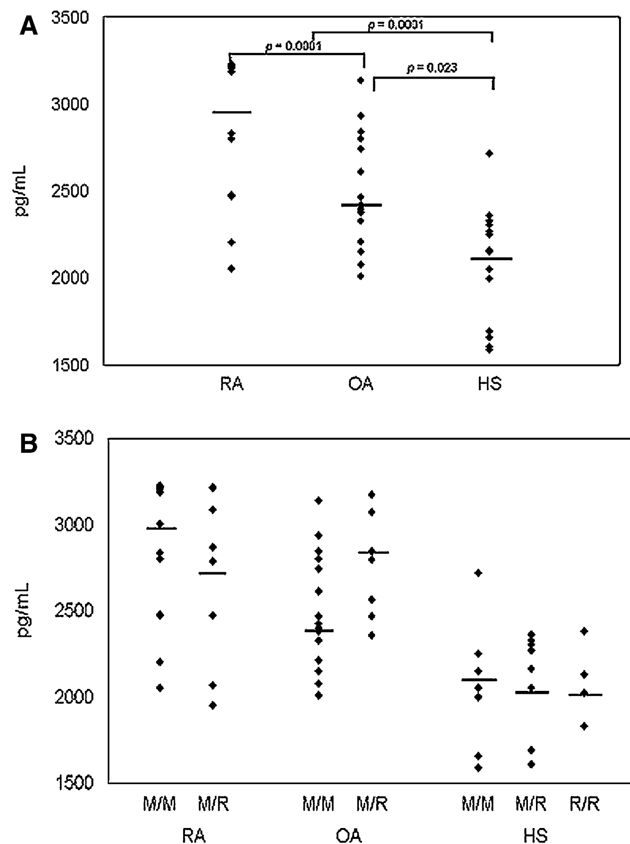

Fig. 1 sTNFR2 levels in RA, OA patients, and HS. *RA* rheumatoid arthritis, *OA* osteoarthritis, *HS* healthy subjects, *M/M* methionine–methionine genotype, *M/R* methionine–arginine genotype, *R/R* arginine–arginine genotype. **a** sTNFR2 serum levels in RA, OA patients, and HS. **b** sTNFR2 levels in RA, OA patients, and HS M/M, M/R, and R/R genotype carriers

It is important to point out that in our study this polymorphism is associated with knee OA. There are no previous reports that indicate this finding. In OA, there is a disturbance in the regulation of synthetic and resorptive activities, in addition pro-inflammatory cytokines like TNFα affects the activities of chondrocytes inhibiting synthesis of proteoglycans and type II collagen, leading to progressive degradation of the cartilage matrix and loss of joint function [24]. TNFα performs its effects by TNFR1 and TNFR2. The M196R polymorphism in receptor 2 could have an effect on TNFα role in dysregulation of chondrocyte function in OA by increases in TNFR2 levels, affecting TNFα binding, and affecting TNFα induced apoptosis and impaired NFκB signaling. Besides that, RA and OA patients, M/M and M/R genotype carriers do not shown differences in the clinical manifestations of the disease.

Elevated levels of acute phase reactants and RF in RA patients with respect to the OA group support the disease clinical status. In this study, differences in TC, TG, LDL-c, apoA-I, and apoB concentrations in RA

patients were found. Changes in lipid profile and acute phase reactants have been associated with early atherosclerosis in RA patients [25], although atherosclerosis and acute myocardial infarction are reported as the most common cause of death in these patients [26]. Reduced apoB levels in RA and SLE patients treated with chloroquine have been reported [27]. OA patients only showed difference in apoA-I and apoB levels with respect to HS. These findings are supported by the fact that, in another study, apoA-I was noted to be protective for myocardial infarction and apoB was a stronger predictor of risk than LDL-c in women and men [28]. Conversely, increased sTNFR2 levels were found in RA and OA patients. This finding correlates with previous data reported by other authors who described elevated sTNFR2 levels in RA patients [10, 29].

In conclusion, the M allele is associated with knee OA. We suggest a lack of association of *TNFR2* M196R in Mexican RA patients. Moreover, sTNFR2 levels are elevated in RA and OA patients but no differences are observed between M/M and M/R genotype carriers.

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