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Association of a *TRAF1* and a *STAT4* gene polymorphism with increased risk for rheumatoid arthritis in a genetically homogeneous population

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Summary Rheumatoid arthritis (RA) is a multifactorial disease that is increasing in incidence worldwide. It is associated with a complex mode of inheritance, with many genes being involved in the development and progression of the disease. Genome-wide association studies in different populations have recently revealed a significant association between a *TRAF1/C5* and a *STAT4* polymorphism and the development of RA. In the present study we performed a case-control study in the population of the island of Crete, Greece, aiming to replicate the former findings in a genetically homogeneous cohort of patients. We found that mutated allele A or genotypes A/A and G/A of the *TRAF1/C5* rs10818488 SNP were more common in individuals with RA than in control individuals (odds ratio [OR] = 1.7, 95% confidence interval [CI] = 1.35-2.15, and OR = 2.22, 95% CI = 1.61-3.05, respectively). Similarly, mutated allele T or genotypes T/T and G/T of the *STAT4* rs7574865 SNP were also associated with susceptibility to RA (OR = 1.9, 95% CI = 1.46-2.50, and OR = 2.37, 95% CI 1.73-2.25, respectively). Thus, we conclude that mutant alleles or genotypes of both polymorphisms examined are associated with the development of RA in our population.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, with a complex mode of inheritance with

many genes being involved in the development and the progression of the disease [1,2]. Genome-wide scans and genetic analyses of various types have been intensively used in the search for genetic determinants of the disease. These studies have mapped the human leukocyte antigen (HLA)-DRB1 [3] gene as well as several non-major histocompatibility complex (MHC) RA susceptibility loci. However only a

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ABBREVIATIONS

HLA	human leukocyte antigen
IL	interleukin
MHC	major histocompatibility complex
PCR	polymerase chain reaction
RA	rheumatoid arthritis
STAT	signal transducer and activator of transcription
TNF	tumor necrosis factor

fraction of these have been validated in independent cohorts so far. Thus, apart from some well-established susceptibility loci such as PTPN22 [4], other promising genes that confer a modest level of risk are currently being investigated. A genomewide association analysis revealed an additional genetic region, the TRAF1-C5 containing locus on chromosome 9, which was associated with an increased risk for RA [5,6]. Recently studies in a North American Caucasian and in a Korean population have documented the association of a common STAT4 haplotype with both RA and systemic lupus erythematosus using a combined positional mapping and candidate-gene approach catalyzed by finding a linkage peak on chromosome 2q [7,8].

The TRAF1/C5 region consists of the TRAF1 (TNF-receptor associated factor 1) and C5 (complement component 5), both of which are immune-related genes that may be involved in the onset and/or perpetuation of inflammation. The TRAF1 gene encodes an intracellular protein that mediates signal transduction through tumor necrosis factor (TNF) receptors 1 and 2 and through CD40. TNF is a critical cytokine in the pathogenesis of rheumatoid arthritis, and TNF antagonists are an effective treatment for rheumatoid arthritis [9,10].

The signal transducer and activator of transcription (STAT) proteins are a family of latent cytosolic transcription factors, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT proteins are activated in response to many cytokines, growth factors, and hormones; after the binding of cytokines or growth factors to their receptors, STAT proteins can be phosphorylated for activation on either tyrosine or serine residues [11,12]. STAT4 is expressed in activated peripheral blood monocytes, dendritic cells and macrophages at sites of inflammation in human beings [13] and lies in the signaling pathway of several important cytokines, including interleukin (IL)-12 and type I interferons, as well as IL-23 [14]. STAT4 mediates IL-12 signaling that is critical for the development of protective immunity in intracellular infection. The mechanism of STAT4-mediated IL-12 signaling in such protection is dependent on the induction of Th1 responses and interferon- γ production [15,16]. Moreover STAT4 regulates T helper 1 (Th1) cell differentiation.

A significant source of variability in the literature of autoimmune diseases has been the inability to replicate genetic findings across the major racial groups, particularly Caucasian and Asian: for example, the association of the intracellular phosphatase PTPN22 with several autoimmune diseases. Thus the possibility that there is true locus heterogeneity for various autoimmune diseases populations of Caucasian European and Asian ancestry should be examined

by performing comparative studies to replicate the association findings across other ethnic or racial groups. Thus comparative studies should be carried out in various populations to replicate the genetic association detected.

In this report we confirm a genetic association in a Greek cohort, as we demonstrated that the two common polymorphisms, the rs7574865 of STAT4 and the rs10818488 of TRAF1 confer a remarkable degree of risk for RA in the population of Crete. Crete is the largest island of Greece, with approximately 0.65 million inhabitants who share the same genetic and cultural background and a common environment.

Subjects and methods**Study population**

The study group comprised 344 healthy subjects and 311 RA patients from unrelated families living in Crete. Age- and sex-matched healthy volunteers from the Department of Transfusion Medicine of the University Hospital of Crete served as controls. All RA patients met the American Rheumatism Association 1987 revised criteria [17]. Ethnic bias within the population studied was minimized by excluding patients that were not of Cretan origin. Cretan origin was defined as having the four grandparents of each individual possessing Cretan ancestry. The study was performed in the laboratory of Internal Medicine, Faculty of Medicine, University of Crete, after obtaining the approval of the research committee and the informed consent of the patients.

Analysis of rs10818488 (TRAF1) and rs7574865 (STAT4) polymorphisms

Whole blood was collected in ethylenediaminetetraacetate (EDTA)-containing tubes. Genomic DNA was isolated from peripheral blood leucocytes by using the commercial kit PUREGENE (Gentra SYSTEMS, Minnesota, MN). The extracted DNA was stored at -20°C until analyzed. After we confirmed by preliminary genotyping that the most significant risk SNPs in the North American and the Korean RA populations, the rs10818488 and rs7574865 of the TRAF1 and STAT4 genes, respectively, were also polymorphic in Cretan population, we proceeded in the genotyping of the cohort of RA patients as well as the proper age- and sex-matched controls.

The upstream primer 5'-GCA GCA GAA CTA CGT GA-3' and the downstream primer 5'-GCT TGC TGT TGA AAT CCT GAA GG-3' were used to generate a region of 226 bp of the TRAF1 gene. Similarly the upstream primer 5'-AAA GAA GTG GGA TAA AAA GAA GTT TG-3' and the downstream primer 5'-CCA CTG AAA TAA GAT AAC CAC TGT-3' were used to generate a region of 147 bp of the STAT4 gene. The amplification was carried out using the GoTaq polymerase provided by Promega. Polymerase chain reaction (PCR) products were analyzed through electrophoresis on 2% agarose gel and ethidium bromide fluorescence in reference to a molecular weight marker.

Genotyping for the TRAF1 rs10818488 SNP was performed by restriction analysis using the S_{dv}I restriction enzyme (Fermentas), which digests specifically DNA amplified from the G (but not the A) allele into 169-bp and 57-bp fragments. Accordingly, the STAT4 rs7574865 PCR product (147-bp) was digested with HpaI (New England Biolabs), which digests the DNA that is amplified from the minor allele (T) into 122-bp and 25-bp fragments. Both undigested and digested PCR products were visualized in 2.5% agarose gel stained with ethidium bromide. Genotypes were scored blindly and analysis of all ambiguous samples was repeated. Moreover a 10% of the samples were amplified twice for checking the accuracy of the results.

Confirmation of polymorphism by direct sequencing

Selected PCR amplified segments, corresponding to A/A and G/G genotypes, were completely sequenced both strands in a LiCor 4200L sequencer of the laboratory of Microchemistry (IMBB-FORTH, Crete, Greece) in order to confirm that the amplified products represent genuine *TRAF1* regions. Similarly, PCR amplified segments, corresponding to G/G and T/T genotypes were completely sequenced to confirm that they represent genuine *STAT4* regions.

Statistical analysis

In the case-control comparisons, only unrelated cases and controls were used. The *TRAF1* and *STAT4* gene variants under investigation were evaluated for deviation from Hardy-Weinberg equilibrium by comparing observed and expected genotype frequencies by means of χ^2 test or Fisher's exact test in the control groups. The statistical difference in genotype distribution and allele frequencies in both control and case subjects was assessed by using standard $2 \times 2 \chi^2$ test or Fisher's exact test when appropriate. Odds ratios (ORs) and confidence intervals (CIs) were calculated. Because one polymorphism was being investigated for each gene, a *p* value of ≤ 0.05 was defined as significant.

Results

The RA study group (*n* = 311) consisted of 172 women and 139 men; unrelated healthy controls (*n* = 344) were of similar age and sex. Mean (\pm SD) age in patients with RA was 61.82 ± 15.11 years. Allele and genotype frequencies of the analyzed samples (311 cases and 344 controls) of the *TRAF1* rs10818488 G/A and *STAT4* rs7574865 G/T polymorphisms are depicted in Tables 1 and 2, respectively. The distribution of genotypes in the control group showed no deviation from Hardy-Weinberg equilibrium (*p* = 0.16). With regard to the *TRAF1* polymorphism, we found that the G/A or A/A genotypes were more common in RA patients (68.2%) than in control individuals (49.1%). The observed difference was statistically significant when it was evaluated with a $2 \times 2 \chi^2$ test of independence (*p* < 0.05). Thus it can be assumed that there is an apparent correlation between mutant genotypes and RA in patients of Cretan origin. The OR rate was used for the evaluation of relative risk (OR = 2.22, 95% CI = 1.61-3.05), indicating that the presence of the aforementioned genotypes contribute to the increase of the disease risk. Patients with RA presented more commonly with A allele

(39.4%) than controls (27.6%) (*p* < 0.05, OR = 1.7, 95% CI = 1.35-2.15). Thus it can be assumed that there is an apparent correlation between the mutant genotypes (G/A or A/A) and RA in patients of Cretan origin.

In the case of *STAT4* polymorphism, G/T or T/T genotype was more common in RA patients (54.3%) than in control individuals (33.0%) who had mostly wild-type G/G genotype (*p* < 0.05, two-tailed χ^2 ; OR = 2.37, 95% CI = 1.73-2.25). Strong deviation from Hardy-Weinberg equilibrium was observed in the distribution of genotypes in the control group (*p* = 0.000438). Similar findings were observed and for the alleles frequencies (G and T) in patients and controls. The T allele was most frequently observed in patients (27.5%) than in controls (16.6%) and this difference was statistically significant when it was evaluated with a $2 \times 2 \chi^2$ test of independence (*p* < 0.05). The presence of this allele increased the disease risk by ~2 times (OR = 1.9, 95% CI = 1.46-2.50). These findings clearly support the implication of the polymorphism under study in the development of RA in Crete, in accordance to previous studies conducted using cohorts of different racial or geographic origin.

Discussion

In recent years, numerous studies have tested association between various candidate genes and the development or progression of RA in patients of different ethnic origin. We present here data showing that variant alleles of *TRAF1/C5* and *STAT4*, conferring an increased risk for RA, are associated also with susceptibility for RA in the population of Crete. These findings are consistent to association data collected from worldwide spread populations and suggest that the polymorphic alleles represent genuine susceptibility alleles. When we examined the association between *TRAF1/C5* or *STAT4* mutated genotypes or alleles in RA patients and the presence of rheumatoid factor, no correlation was found as the differences were not statistically significant (data not shown). Interestingly, the frequency of the A allele of the *TRAF1/C5* SNP was lower in the healthy controls from Crete (27.6%) as compared with either the Dutch (40%) [5] or Spanish controls (36%) (Kurreeman *et al.*, unpublished data). We also observed a much lower frequency of the T allele of the *STAT4* SNP in the healthy individuals from Crete (16.6%) as compared with the North American NARAC cohort (22%) [7]. Taken together, these findings indicate that there are distinct population specific differences in the prevalence

Table 1 Genotypes and alleles frequency of the *TRAF1* G/A polymorphism analyzed in 311 rheumatoid arthritis (RA) patients and 344 healthy controls.

Allele frequency	Mutated allele A (%)	Wild-type allele G (%)	<i>p</i> value*	OR (95% CI)*
RA subjects (<i>n</i> = 622)	245 (39.4)	377 (60.6)	0.000006	1.7 (1.35-2.15)
Control subjects (<i>n</i> = 688)	190 (27.6)	498 (72.4)		
Genotype frequency	Mutated A/A or A/G (%)	Wild-type G/G (%)		
RA subjects (<i>n</i> = 311)	212 (68.2) ^a	99 (31.8)	0.000001	2.22 (1.61-3.05)
Control subjects (<i>n</i> = 344)	169 (49.1) ^b	175 (50.9)		

OR, odds ratio; CI, confidence interval.

* *p* values and ORs for differences in allele A frequencies between case and control subjects.

^a Includes 179 genotypes A/G and 33 genotypes A/A.

^b Includes 148 genotypes A/G and 21 genotypes A/A.

Table 2 Genotypes and alleles frequency of the STAT4 G/T polymorphism analyzed in 311 rheumatoid arthritis (RA) patients and 344 healthy controls.

Allele frequency	Mutated allele T (%)	Wild-type allele G (%)	<i>p</i> value*	OR (95% CI)*
RA subjects (<i>n</i> = 622)	171 (27.5)	451 (72.5)	0.00002	1.9 (1.46-2.50)
Control subjects (<i>n</i> = 688)	114 (16.6)	574 (83.4)		
Genotype frequency	Mutated T/T or T/G (%)	Wild-type G/G (%)		
RA subjects (<i>n</i> = 311)	168 (54.3) ^a	143 (45.7)	0.00	2.37 (1.73-2.25)
Control subjects (<i>n</i> = 344)	114 (33) ^b	230 (67)		

OR, odds ratio; CI, confidence interval.

* *p* values and ORs for differences in allele A frequencies between case and control subjects.

^a Includes 165 genotypes T/G and three genotypes T/T.

^b Includes 114 genotypes T/G and no genotypes T/T. The absence of T/T genotype in the control group was also statistically significant when compared with patients group (*p* = 0.029).

of these alleles. Of note, the same alleles that predispose for RA also predispose to other autoimmune disorders such as systemic lupus erythematosus, Sjögren's syndrome [7,18], and Wegener's granulomatosis and type 1 diabetes (Goulielmos *et al.*, unpublished data), thus suggesting that the regions under examination may contribute to shared molecular pathways that lead to multiple autoimmune diseases. However the possibility that some additional alleles of these two chromosomal regions are involved in the pathogenesis of these diseases should be clarified by further fine mapping of these regions.

The rs7574865 SNP examined is located in the third intron of the STAT4 gene, and its actual functional consequence remains to be identified. In a sequence analysis performed in our laboratory, by using the Genomatix (Genomatix Software GmbH, Gene2Promoter program, Munchen, Germany), the polymorphic site was not found to disrupt any transcription factor binding site. To obtain further insight into this genetic pathway that is shared among various autoimmune diseases, we currently assess the phosphorylation levels of STAT4 as well as the cytokine production in subjects of different STAT4 genotypes. In contrast, the TRAF1 rs10818488 SNP is located within a region encoding a putative binding site for the transcription factor P300. P300 frequently plays important roles in a broad spectrum of biologic processes, including cell proliferation and differentiation, and has been described as a transcriptional co-activator to reflect its ability to associate with a variety of cellular and viral transcription factors including nuclear hormone receptors, CREB, AP-1, and E1a, as well as with other co-activator proteins [19]. Therefore, aiming to assess the effect of TRAF1 mutant genotypes in RA pathogenesis, assays for differential binding of the transcription factor may be designed by involving samples from the three different genotypes.

Pathogenesis of RA is very complex; therefore any of the involved factors such as immune cells, mediators (including cytokines and chemokines), genetic background, and environmental conditions may alter the disease outcome. Therefore, conflicting studies in several cases have made the interpretation of these data challenging. Discrepancies could represent differences in the genetic background between populations studied, small sample sizes or inadequate definition of phenotypes. A definite advantage of our study, particularly with respect to other association studies, was the attention paid to the selection of a genetically and ethnically homogeneous patient cohort and control group. As a

consequence, the results of this study are unlikely to be biased by sampling. A possible weakness of our study deals with the limited sample size, a fact that is difficult to be overcome easily in a geographical isolated region. However the limitation previously mentioned may reflect an advantage in that, in such a geographically isolated gene pool, alleles of low frequency that could be undetectable in a larger population, may be detected in our case [20]. Further investigation is needed to clarify the putative functional significance of the polymorphisms under examination and any possible genetic linkages with other polymorphisms of known functional effect.

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