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Lack of Association between Single Nucleotide Polymorphism rs10818488 in TRAF1/C5 Region and Rheumatoid Arthritis in Iranian Population

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ABSTRACT

The association of rs10818488 SNP located in TRAF1/C5 region with Rheumatoid Arthritis (RA), has been picked up by genome-wide association studies. Independent studies in different populations revealed inconsistent results. The aim of this study was to investigate the possible association of this SNP with RA in Iranian population.

A total of 362 cases and 422 healthy controls were recruited in this study. Genomic DNA was extracted from whole blood and the genotyping was performed by PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism). A set of genotypes was confirmed by sequencing. Genotype and allele frequencies were compared between the case and control groups.

Analysis indicated a higher frequency of A allele in cases, although the difference was not statistically significant (Chi-square=2.8, p=0.09). Comparison of genotype frequencies, revealed higher frequencies of AA and AG genotypes in case group but statistically the difference was not significant (Chi-square=2.72, p=0.25).

These findings suggest that the rs10818488 in TRAF1/C5 region is not associated with rheumatoid arthritis in Iranian population.

Keywords: Association study; C5; Rheumatoid arthritis; Single nucleotide polymorphism (SNP); TRAF1

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disorder with overall prevalence of 1% in adult population. RA has a complex etiology. Many genetic elements as well as environmental factors contribute to the individual susceptibility to RA. Previous studies showed an estimated heritability of 50-60% for RA.[1] The frequency of RA in Iranian population was reported to be 0.33%. [2]

Recent genome-wide association study, using RA samples from the North American Rheumatoid

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Arthritis Consortium (NARAC) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis, picked up several susceptibility loci for RA including the TRAF1/C5 region on chromosome 9p33.2. The association of this region with RA was confirmed in another independent study of Caucasian subjects from Dutch population using the candidate gene approach. In this study 40 SNPs in TRAF1/C5 region were investigated and the rs10818488 was identified as the most strongly associated SNP in the TRAF1/C5 region with RA.[3-4] Other case-control and family-based association studies in European decent, repeatedly validated TRAF1/C5 region as one of RA risk loci in Caucasian population.[5-7] These studies suggested TRAF1/C5 region as the third major locus responsible for genetic risk to RA after HLA-DRB1, and PTPN22 gene.[3,4]

The TRAF1/C5 region contains the TNF receptor associated factor 1 (TRAF1) as well as complement component 5 (C5) genes. The TRAF1 gene encodes an intracellular protein that mediates signal transduction through TNF receptor 1 and 2 and through CD40. TNF is a critical cytokine in the pathogenesis of RA and TNF antagonists are effective treatments for RA.[8,9] On the other hand, C5 is a member of complement system and codes for complement component 5 which could contribute to the development of RA through tissue destruction and the mobilization of inflammatory and synovial cells.[10] Complement components and regulatory molecules have been implicated in the pathogenesis of RA and are present in synovial tissues. A critical role of complement in the inflammatory process makes C5 a plausible candidate gene in this region.[11]

TRAF1/C5 region is associated with several autoimmune diseases besides RA, including juvenile idiopathic arthritis (JIA) and systemic lupus erythematosus (SLE),[12,13] implying that this region lies in a pathway relevant to multiple autoimmune diseases.

RA is a complex disorder and several factors would complicate the molecular genetics of RA, such as population stratification, environmental factors and genetic heterogeneity. DNA polymorphisms clearly show different allele and genotype frequencies in different populations. On the other hand, environmental factors and their interactions with genes have different effects in different populations. This means that the results obtained in genetic-based association studies in one population cannot be extended to other populations before repeating independent studies. Therefore, we decided to investigate the possible association of rs10818488 with RA in Iranian population.

MATERIALS AND METHODS

In this study, a case-control design was used to test the possible association of rs10818488 with RA. In total, 823 individuals consisting of 380 patients and 443 controls were recruited for the study. Thirty-nine samples were removed from the final analysis due to the incomplete data and suspicious genotypes. Finally 784 samples consisting of 362 patients and 422 controls with no history of RA or other autoimmune diseases were included in our study population.

All patients met the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA [14]. The control subjects were recruited from patient's spouse or healthy individuals visiting the hospital for other reasons. All study subjects were of Iranian origin and gave informed written consent before entering to the study. The local committees at the Tehran University of Medical Sciences approved the study in terms of ethical and legal requirements.

Effort was made to equalize the proportion of different Iranian ethnic groups in case and control groups. Demographic features of the population under study are presented in Table 1.

Experimental Procedures

Whole blood was collected in ethylenediamine tetra acetic acid (EDTA) containing tubes. Genomic DNA was isolated, using standard phenol/chloroform extraction, ethanol precipitation method. The quality and quantity of genomic DNA were determined by agarose gel electrophoresis and UV spectrophotometer measuring absorption at 260 and 280 nm wavelengths. DNA was stored at 4 ˚C until analysis.

Genotyping was performed by PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technique. A set of genotypes was confirmed by direct DNA sequencing. The upstream primer 5'- ACA GGA GCC AGA GTG AGC AG -3' and downstream primer 5'-GCA GCA GCA GAA CTA CGT GA -3' were used to amplify the region of interest. The polymerase chain reaction (PCR) was carried out in a total volume of 25µl

containing 100-200 ng of genomic DNA, 0.5 mM of each primer, 2 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 units Taq DNA polymerase.

Amplification was performed with a denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 45 seconds. The final extension step at 72°C was done for 5 min. Three to five µl of PCR products were run on 1% agarose gel to see the target band before performing the restriction digestion reaction. Digestion was performed in a total volume of 20 µl, containing 15 µl of PCR products, 5 units of Sdu1 enzyme (10 Units/µl, Fermentas Inc.), 2 µl 10x enzyme buffer, and 2.5 µl of double distilled H_2o. Digestion reaction was incubated at 37°C overnight. Digested products were analyzed by running on 3% agarose gel next to ultra low range DNA ladder (Fermentas), stained by ethidium bromide, visualized under UV light and photographed.

Direct sequencing of PCR products was performed by Pishgam Biotech Company, Tehran, Iran. Standard dideoxy sequencing method was used in Applied Biosystems 3730x Genetic Analyzer (Figure1a, b, c).

Statistical Analysis

To test the Hardy-Weinberg equilibrium in the study groups, chi-squared test was performed to compare the observed and expected genotype frequencies in cases and controls. To compare the allelic and genotype frequencies among cases and controls, again chi-squared test was performed. Using SPSS version 17 and a *p* of 0.05 or less was defined as significant.

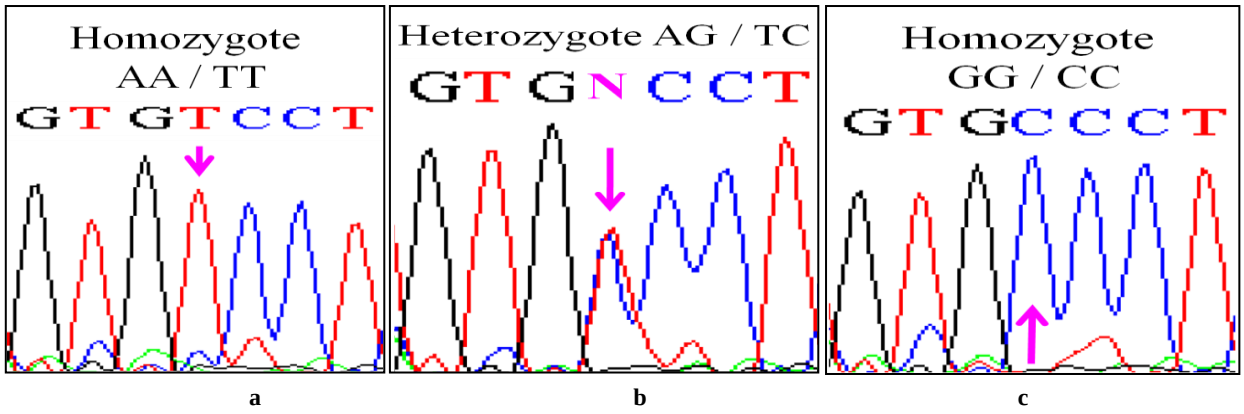

Figure 1. Sample sequencing results for PCR products showing the rs10818488 and its flanking nucleotides. a) homozygote AA (TT on complementary strand), b) heterozygote AG, (TC on complementary strand), c) homozygote GG (CC on complementary strand).

Table 1. Demographic features of case and control groups.

Groups Number / Proportion		Female	Male
Case group (number/percent)		297 / 82%	65 / 18%
	Age (Year)	45.7 ± 12.9	51.6 ± 13.9
	Height (cm)	158.8 ± 6	172.4 ± 6
	Weight (kg)	69.1 ± 12	74.5 ± 12
Control group (number/percent)		201 / 47.6%	221 / 52.4%
	Age (Year)	57.3 ± 10	60.8 ± 11
	Height (cm)	157.1 ± 6	168.8 ± 7
	Weight (kg)	71.8 ± 12	75.2 ± 12

All variables are expressed as mean ± SD.

Table 2. Genotype distribution and allele frequencies of rs10818488 in RA patients and controls.

Groups	Genotypes: Number / Frequency				Alleles: Number / Frequency			Hardy-Weinberg	
	AA	AG	GG	Total	A	G	Total	χ^2	*p*
Case	52 / 0.14	160 / 0.44	150 / 0.41	362 / 1	264 / 0.37	460 / 0.63	724 / 1	0.66	0.42
Control	47 / 0.11	180 / 0.42	195 / 0.47	422 / 1	274 / 0.32	570 / 0.68	844 / 1	0.82	0.37
Total	99 / 0.13	340 / 0.43	346 / 0.44	784 / 1	538 / 0.38	1030 / 0.62	1568 / 1		
	χ^2 = 2.72, *P*= 0.1, df = 1				χ^2 = 2.82, *P*= *0.09*, df = 1				

RESULTS

Demographic features of case and control groups were summarized in Table 1. PCR amplification of target fragment was performed successfully and the desired product of 274 nucleotides was seen on agarose gel (Figure 2). The amplified fragments contain one recognition site for the SduI enzyme (5'- G (G/A/T) GC (C/T/A) C - 3'), independent of the rs10818488 SNP. We included this stable site on the amplified products to be used as internal control to show the successful complete digestion of the products. Cutting the PCR products at this site, separates a fragment of 38 bp from the products. This fragment must be seen on the gel for all digested products. On the other hand, presence of G allele at the polymorphic site, creates another recognition sequence for the SduI enzyme that following digestion, produces two other fragments of 165 and 71 bp. Therefore, an amplified fragment with G allele produces three smaller fragments of 165, 71 and 38 bp following digestion. A fragment with A allele was cut into two fragments of 236 and 38 bp. Considering all this, samples with AA genotype produced two bands of 236 and 38 bp on the gel following electrophoresis of digestion products. The same way, samples with GG genotype produced three bands of 165, 71, and 38 bp, and samples with AG genotype produce four bands of 236, 165, 71, and 38 bp (Figure 3).

Following genotyping by PCR-RFLP, a set of samples was genotyped by PCR-sequencing. All genotypes were consistent using both methods.

Table 2 represents the allele and genotype frequencies obtained in our study. Examining the Hardy-Weinberg equilibrium in both case and control groups showed no significant deviation, revealed by χ^2= 0.66 and p = 0.42 in cases and χ^2= 0.82 and p=0.37 in control group.

Comparison of allele and genotype frequencies in case and control groups showed over-representation of A allele as well as higher frequencies of AA and AG genotypes in case group. Comparing allele frequencies in two gene pools produced a $\chi^2 \approx 2.82$ and p= 0.09. On the other hand comparing genotype frequencies between two groups showed a $\chi^2 \approx 2.72$ and $p \leq 0.1$ (Table 2).

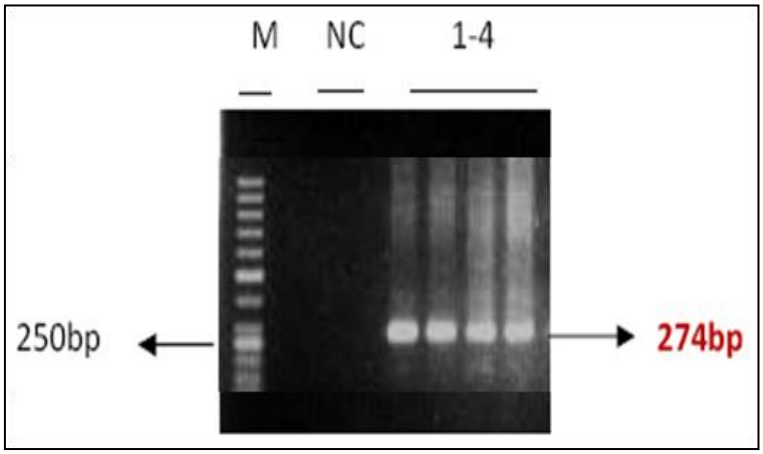

Figure 2. PCR products using template genomic DNA from samples 1 to 4. M: 100-bp DNA ladder, NC: negative control for PCR.

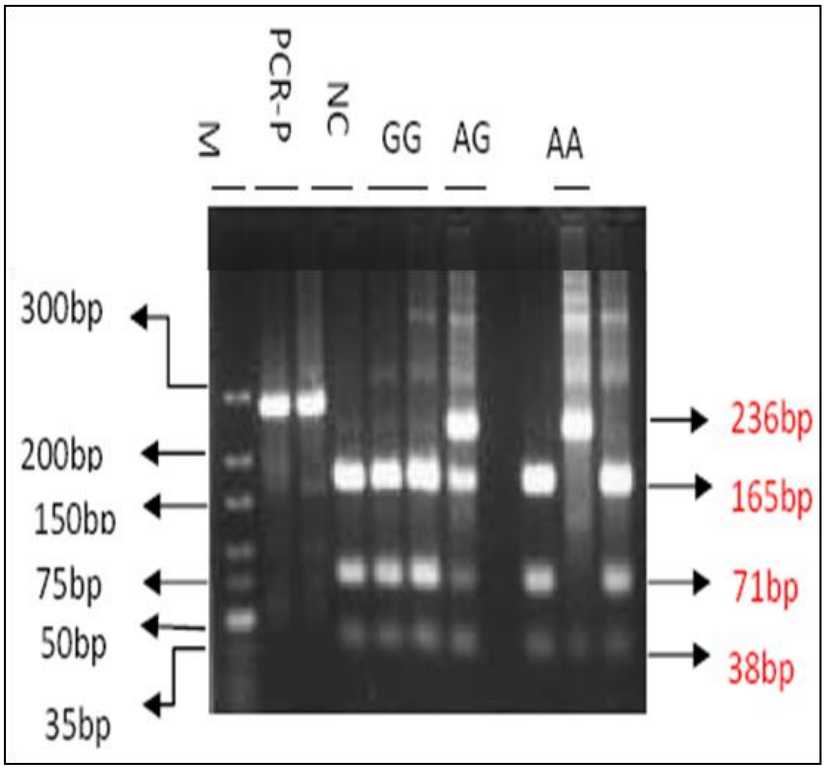

Figure 3. Digestion of PCR product with Sdu1 restriction Enzyme. Lane M contains ultra low range ladder from Fermentas, lane 2 (PCR-P) contains undigested PCR products. Lane 3 (NC) contains negative control (digestion reaction without enzyme), lane 4-10 represent samples with GG, AG and AA genotypes. Arrows show the size of different bands seen on the gel.

DISCUSSION

Association of TRAF1/C5 region with RA was first reported by a genome-wide association study in 2007.[4] Participants in the above-mentioned study were from two data sets, the North American rheumatoid arthritis Consortium and Swedish Epidemiological Investigation of RA. This finding was followed by a number of independent studies in different populations.

A candidate gene approach by Kurreeman et al, aiming to narrow down this region, identified rs10818488 with strongest association with RA. Following studies in different populations with European decent resulted in the same outcome, showing association between rs10818488 minor allele A and RA.[3] A more recent study showed the association of A allele at this SNP with development as well as severity of RA in Egyptian population.[15] Consistent with the above reports, a large meta-analysis confirmed the association between rs10818488 and RA in European and North African populations.[16]

Similar studies did not confirm association of this SNP with RA in Colombian population.[17] The Colombian study was consistent with the first genome-wide association study regarding RA, performed by the Welcome Trust in 2007, that failed to detect association of TRAF1/C5 region with RA.[18] Interestingly, a study by Nishimato et al in Japanese population reported the association of G allele at this SNP with RA.[19]

Our work, did not confirm the association of rs10818488 and RA, although higher frequency of A-allele was observed among patients. Another association study testing the association of this SNP with RA in western Iran was published recently. The study was performed in a very small number of samples (50 cases and 50 controls) and reported no association between the rs10818488 and RA.[20]

Considering inconsistent results obtained in studies on different populations, our result cannot be named as unexpected. There are several explanations for such inconsistent results including false positive or false negative results, population stratification and different pattern of linkage disequilibrium (LD) in different

populations. In other words, it is possible to assume that the real causative DNA variation in this region is not rs10818488 itself, but another variation with linkage disequilibrium with different alleles of rs10818488 in some populations.

Repeating association studies to test other SNPs in this region with RA is needed to confirm above hypothesis and to find the causative DNA polymorphism in this region resulting in susceptibility to RA. Unfortunately, due to lack of financial resources, we were not able to continue with testing other SNPs in this region.

Last but not least, population stratification among samples from Iranian population is an important factor that affects such association studies in Iran. Iranian population consists of many ethnic groups with possible different gene pools. This fact is behind many inconsistent results, obtained in association studies of multifactorial diseases in our country.

It appears that performing family-based association studies instead of population-based studies would gain more reliable results in our population.

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