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Lack Evidence Between CTLA-4 Gene Polymorphisms Among Rheumatoid Arthritis Patients

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Abstract
Rheumatoid arthritis is a chronic inflammatory disorder that typically affects the small joints in hands and feet. Genes involved in T-cell regulation are potential candidates. Association to cytotoxic T-lymphocyte-associated-4 (CTLA-4) protein, a negative regulator of T-cell activation, has previously been described in Rheumatoid arthritis patients. In this study, our aim was to determine the role of CTLA-4 polymorphism among patients with Rheumatoid arthritis. A total of 120 Rheumatoid arthritis patients (69 males and 51 females) with an age mean ± SD (67.2000± 11.74133), family history (16 positive/ 104 negative), rheumatoid factor test (RF) (43 positive/ 77 negative), Anti-CCP antibody test (120 positive/ 0 negative), and 120 controls (46 males and 74 females) of Iraqis ethnicity with an age mean ± SD (31.6583± 11.51579). There was no significant difference between the groups (Rheumatoid arthritis patients and their control) (P = 0.478). And CTLA-4 gene polymorphism in each group was compared (AA, GG, AG). There is no difference between the CTLA-4 (SNP +49 A/G rs#231775) gene polymorphism among Rheumatoid arthritis patients and healthy people.
Keywords: CTLA-4, Genotype, Rheumatoid arthritis, Genetic susceptibility, SNP

Introduction

Rheumatoid arthritis (RA) is a complex autoimmune disorder, characterized by a chronic T-cell response that has evaded normal control mechanisms (GORONZY and WEYAND 2004). Also is characterized by chronic inflammation of the joint, which may lead to structural damage of the cartilage and bone (SINGAL *et al.* 1999). Actually in RA both environmental and genetic factors contribute to the disease, with the later being a substantial contributory factor in the pathogenesis of the disease (JOHN and WORTHINGTON 2001; MACGREGOR *et al.* 2000). A many genes contributed to RA susceptibility has been found, among all these genes, HLA-DR loci was most thoroughly studied yet HLA-DR accounts only for approximately one-third of the genetic susceptibility to RA. Other loci also implicated in the predisposition to RA, such as CTLA4, PADI4, PTPN22, TNF-a, IL-1b and STAT4, account for relatively small additional contribution RA genetic susceptibility (KURKO *et al.* 2013). It has been reported that CTLA-4 polymorphisms are associated with several T-cell mediated autoimmune diseases, such as Graves' disease, type 1 diabetes mellitus (T1D) and multiple sclerosis (KANTARCI *et al.* 2003; KOUKI *et al.* 2002; MARRON *et al.* 2000). The CTLA-4 encodes the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis (WATERHOUSE *et al.* 1995). The human CTLA-4 gene was mapped to chromosome 2q33 (DARIAVACH *et al.* 1988). Most molecular epidemiology studies have evaluated the role of the + 49A/G (rs#231775) single nucleotide polymorphism (SNP) that causes a threonine-toalanine substitution in codon 17 and is associated with altered protein expression (ANJOS *et al.* 2002). The aim of this study was to examine the role of Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) +49 gene in genetic susceptibility as a risk factors for rheumatoid arthritis (RA) in a sample of Iraqis patient.

Materials and methods

120 patients with an established diagnosis of RA, (69 males and 51 females) with an age mean ± SD (67.2000± 11.74133), family history (16 positive/ 104 negative), rheumatoid factor test (RF) (43 positive/ 77 negative), Anti-CCP antibody test (120 positive/ 0 negative). and 120 controls (46 males and 74 females) of Iraqis ethnicity with an age mean ± SD (31.6583± 11.51579), were enrolled in this study and recruited at Baghdad teaching hospital, medical city, Baghdad, Iraq. patients with RA [diagnosed based on American Rheumatism Association (ACR) 1987 Criteria (ARNETT *et al.* 1988). The ethics committees of participating universities and university hospitals approved the study, and informed consent was obtained from all participants. Blood sampling (one ml of venous blood) was collected in EDTA tubes from each individual (patient or healthy control) and was stored as whole blood at -20oC for subsequent DNA isolation. Genomic DNA was isolated from whole blood according to Sambrook et al 1989 (SHUBEITA *et al.* 1987).

Genotyping of (CTLA-4) +49 gene polymorphism

One SNP (rs#231775 A/G) CTLA-4 gene was genotyped among the participants groups in this study. The CTLA-4 A/G polymorphic region (rs#231775 A/G) was amplified by polymerase chain reaction (PCR) using allele specific PCR technique as shown in Table 1. Three primers (two allele specific primers and common reverse primer) were designed based on the nucleotide sequence of a partial fragment (retrieved from the online dbSNP) of the gene containing the target SNP. The polymorphism was visualized by separating the DNA fragments in a 2% agarose gel that was stained with ethidium bromide and illuminated by UV. To validate the PCR- allele specific results to validate the PCR- allele specific results. All primers used in this study were newly designed using Primer Blast online programme (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

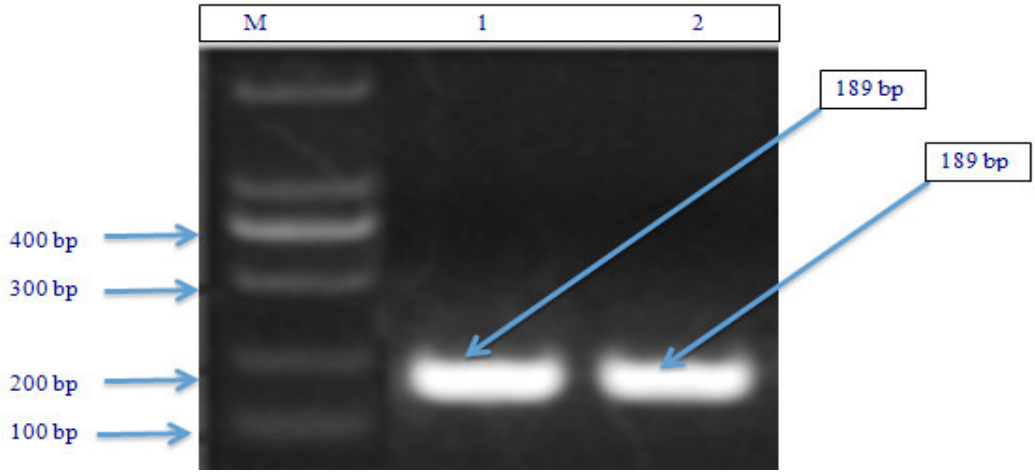

Figure 1:2% agarose gel electrophoresis for allele specific PCR for CTLA-4 gene (rs#231775 A/G) M: 100 bp DNA ladder from GeneDireX®. Lanes 1 and 2: PCR products upon using allele specific C primer and allele specific T primer, respectively. Heterozygous genotype will give positive reaction upon using both allele specific primers. However, homozygous genotype will give positive reaction upon using only one of these allele specific primers.

Table 1: Primers sequences, PCR conditions, length of PCR products

SNPs	Primers sequences	PCR Conditions	Size of PCR Products digestion products
CTLA-4 SNP49 G/A (rs#231775)	A-allele specific primer: F1: 5-GCTCAGCTGAACCTGGCT A -3 G-allele specific primer: F2: 5-GCTCAGCTGAACCTGGCT G-3 Common reverse primer: 5-CTTTAACTTCTGGCTTTGCTAT-3	An initial denaturation at 95°C for 5 min -Then, 30 cycles each cycle consisted of denaturation at 94°C for 60s, annealing at 50 °C for 30s and extension at 72°C for 30 s. -A final extension at 72°C for 10min.	Allele A: 189 bp Allele G: 189 bp

Statistical analysis of data

Statistical analysis of data was done to correlate genotype distribution and allele frequencies were performed by SPSS package version 17. The frequencies of alleles, genotypes in different groups were compared using the Chi-squared test (X2), t-test were used to test the significance of results of quantitative variables. Odds ratio and 95% confidence interval (95% CIs) were calculated for different studied parameters. The confidence interval (CI) at 95% was used to describe the amount of uncertainty associated with the samples (GREENFIELD *et al.* 2008; SZUMILAS 2010). The significance of the results was taken at the $P < 0.05$ level of significance.

Results and Discussion

Results revealed that the allele and genotypic distributions did not significantly differ between the two groups (P>0.05) for the SNP- *CTLA-4* +49 A/G (rs#231775) as shown in table 2.

Table 2: CTLA-4 gene polymorphism and allele frequencies among RA patients and their control

Gene polymorphism	Cases		Control		Significance	OR (95% CI)
	No.	%	No.	%		
CTLA-4 SNP49 G/A (rs#231775)						
AA	25	26.6	49	40.9	$X^2 = 0.504$ P = 0.478	(0.761 - 1.729)
GG	49	40.9	24	20.0		(0.578 - 1.314)
AG	39	32.5	47	39.1		

X^2: Chi-Square test *significant at P≤0.05

The RA is a T-cell-mediated autoimmune disease (LORBER *et al.* 1994). Many genes discovered contributed in autoimmune diseases in murine models (VYSE and TODD 1996). Some genes determine the susceptibility of a target organ to an immune response. CTLA-4 is a prime candidate autoimmunity gene leading to alteration in function could have profound effects on the immune system. Defective CTLA-4 expression could result in failure to terminate T-cell activation (BARTON *et al.* 2000).

Our results corresponded with Barton, A. et al. Noted that no significant differences in the frequency of the G allele or the GG genotype were found in either the UK or Spanish RA patients compared with controls (BARTON *et al.* 2000). Previous study approved that the CTLA-4 gene is associated with Japanese patients with RA carrying the susceptible HLA allele; AG genotype occurred more frequently in patients with RA in Japanese population (59 vs 44%) (YANAGAWA *et al.* 2000).This result agree with Lee, C.S. et al. that suggested the CTLA4 49 A-G polymorphism is associated with RA in Chinese patients from Taiwan, genotype CTLA4 49 G/G and allele G were associated with an increased risk of RA, whereas genotype A/G and allele A were associated with protection against RA (LEE *et al.* 2003).

A total of 30 case-control studies in 20 articles were included in this meta-analysis study found that the +49A/G and CT60 polymorphisms in the CTLA-4 gene may be risk factors for RA according to the results and that showed the variant G allele carriers (GG + GA) of +49A/G polymorphism had an 18% increased risk of RA when compared with the homozygote AA.

In addition, the variant CT60A allele carriers of CT60 polymorphism had a 14% decreased risk of RA when compared with the homozygote GG. In the subgroup analysis by ethnicity, significant elevated RA risks were associated with +49G allele carriers in Asians, but not in Europeans. However, for CT60 polymorphism, significant decreased RA risks were associated with CT60 A allele carriers in Europeans, but not in Asians(LI *et al.* 2012). Other meta-analysis corresponded with previously meta-analysis studies, which suggest CTLA-4 A49G polymorphism was associated with RA risk. The researchers attempted to perform an updated meta-analysis of available case–control study in order to assess the association between CTLA-4 A49G polymorphism and RA risk by searched the various citation databases, totally compiled 27 studies in 24 articles (9805 RA patients and 10691 control subjects) in this meta-analysis work. A significant association between CTL-A4 A49G polymorphism and RA risk (GG vs. AA: OR = 1.13, 95% CI = 1.03–1.23; GA vs. AA: OR = 1.19, 95% CI = 1.07–1.33; GA + GG vs. AA: OR = 1.18, 95% CI = 1.07–1.29). In the subgroup analysis by ethnicity, evidences of significantly increased risk was also found in both Asian (GG vs. AA: OR = 1.34, 95% CI = 1.15–1.55; GA + GG vs. AA: OR = 1.24, 95% CI = 1.08–1.41) and Caucasian population (GA vs. AA: OR = 1.19, 95% CI = 1.03–1.37; GA + GG vs. AA: OR = 1.14, 95% CI = 1.01–1.29) (LI *et al.* 2014) .

Our conclusion, we found there is no association between CTL-A4 A49G gene polymorphism and RA among Iraqis population.

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