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Single Nucleotide Polymorphism of *Padi4* Gene (Rs11203367) in A Sample of Rheumatoid Arthritis Iraqi Patients

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Abstract

Rheumatoid arthritis (RA) is an inflamed chronic autoimmune disease in which genetics and environment are the most common causative factors. Peptidyl arginine deiminase type IV (PADI4) is an enzyme responsible for the posttranslational conversion of arginine residues into citrulline. Real-time polymerase chain reaction (RT-PCR) is a specific technique was used to determine gene polymorphism. One hundred twenty three patients molecularly confirmed with RA and sixty healthy control subjects were recruited. By applying the logistic regression analysis, some alleles and genotypes were associated with susceptibility to RA. Under the allelic model, C allele frequency was significantly increased in RA patients compared to control (58.5 vs. 46.7%; p = 0.034). The odds ratio (OR) of such difference was 1.61 (95% CI: 1.04 – 2.50). Under the dominant model, TC+CC genotype frequency was higher in patients than in control (82.1vs. 65.5%; OR = 2.47; 95% CI: 1.23 – 4.97; p = 0.015). Under the codominant model, frequency of the heterozygous genotype TC was significantly increased in patients compared to control (47.1 vs. 36.7%; OR = 2.52; 95% CI: 1.17 – 5.41; p = 0.028). The results revealed the role of the rs11203367 variant of *padi4* gene in susceptibility to RA in Iraqi patients.

Keywords: Rheumatoid arthritis, Peptidyl arginine deiminase type IV, polymorphism, Real time PCR.

تعدد اشكال النيكليوتيدة المفردة لجين *padi4* (rs11203367) في عينة من مرضى التهاب المفاصل الرثوي العراقيين

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الخلاصة

يعتبر التهاب المفاصل الرثوي (RA) مرض مناعي ذاتي مزمن يمكن أن تشارك فيه العوامل الوراثية والبيئية. يقوم إنزيم Peptidyl arginine deiminase type IV (PADI4) بتحويل بقايا الأرجينين بعد الترجمة إلى سيترولين. تم تحديد تعدد الأشكال عن طريق تفاعل البلمرة المتسلسل في الوقت الحقيقي (RT–PCR). وفقًا لذلك ، تم إجراء دراسة على 123 مريضًا بمرض التهاب المفاصل الرثوي و 60 حالة سيطرة. اظهر تحليل الانحدار اللوجستي ارتباط بعض الأليلات والأنماط الجينية بالتعرض لمرض التهاب المفاصل الرثوي. تحت النموذج الأليلي ، زاد تردد أليل *C* بشكل ملحوظ في مرضى التهاب المفاصل الرثوي مقارنةً بمجموعة السيطرة (58.5 مقابل 47.7%; p= 0.015). كانت نسبة الأرجحية (OR) لهذا الاختلاف 1.61 (CI 95%: 1.04 – 2.50). تحت النموذج السائد ، تبين ان تردد النمط الجيني TC + CC أعلى في المرضى منه في

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السيطرة (82.1 مقابل 65.5%; p = 0.015 ; 95% CI: 1.23 – 4.97 ; OR =2.47). لوحظ في النموذج المشفر ، ازدياد تواتر النمط الجيني متغاير الزيجوت TC بشكل ملحوظ في المرضى مقارنة بمجموعة السيطرة (47.1 مقابل 36.7% ; p = 0.028 ; 95% CI: 1.17 – 5.41 ; OR = 2.52). اظهرت النتائج اعلاه ان هنالك دور لتعدد اشكال النيوكليوتيدة المفردة rs11203367 (لجين *padi4*) في القابلية للإصابة بمرض التهاب المفاصل الرثوي في المرضى العراقيين.

Introduction

Rheumatoid Arthritis (RA) is considered the most common autoimmune disorder that results in a significant morbidity rate [1]. This multifactorial disease is affecting up to 1% of the adults in the population worldwide. It occurs in different age ranges and more prevalent in women than men. RA causes the destruction of the synovial joints, prompting serious dysfunction and premature mortality [3, 4]. Women are three times more susceptible than men and the disease is more frequent at the age range 40–50 years [5, 6]. Disease severity is measured often by the development of joint destruction. The major known risk factors include inflammation and development of autoantibodies, which are speculated to explain 32% of the variance in joint destruction [7]. Early diagnosis in addition to early and effective therapy are demanded to prevent joint damage and lead to better long-term results. Therefore, reliable biomarkers along with outcome measures are needed [5]. Genetic background and environmental factors are the affective factors in RA pathogenesis, etiology, prognosis, and outcomes [8]. The cause of RA is unknown, but substantial evidence suggests that the disease develops in individuals after interaction between inherited genetic risk factors and environmental triggers. Such interaction can lead to immune dysfunction that is identified by the presence of autoantibodies and disturbance of cytokines in the serum, many years prior to the diagnosis of the disease [9]. The Peptidyl Arginine Deiminase genes family, including the 4 *padi4* gene, encode for enzymes that are engaged in the posttranslational conversion of arginine residue to citrulline [10].

The generation of Anti-Cyclic Citrullinated Peptide (Anti-CCP) antibodies that are highly specific in RA is mediated by PADI enzymes [11]. The detection of Anti–Citrullinated Protein Antibodies (ACPA) by different methods has been used for decades as a diagnostic tool of RA since the discovery of the pivotal role of citrullination in the disease [12]. The present study aims to evaluate the association between rs11203367 polymorphism of *padi4* gene and RA among Iraqi patients.

Materials and methods

The present study involved 123 Iraqi RA patients (31 males and 92 females) whose age range was 20 to 50 years. They were referred to the Rheumatology Clinic at Baghdad Teaching Hospital during September 2019 – February 2020 for diagnosis and treatment. The diagnosis was made by the consultant medical staff at the Rheumatology Unit and based on the revised diagnostic criteria ingrained by the American College of Rheumatology (ACR), 2010. All patients received a comprehensive description of the study and gave written informed consent for their participation. In addition to the patients, 60 healthy controls, who underwent the necessary tests to ensure their healthiness and the absence of chronic diseases and infections, were also enrolled in the current study. While for patients, laboratory tests were conducted to confirm the diagnosis of disease.

Blood samples (3 ml) from patient and control groups were collected in EDTA tubes for DNA extraction. The total DNA was isolated from the whole blood = using DNA extraction kit for ReliaPrep genomic DNA Minprep System (Promega/USA). The extracted DNA was precipitated by the salting out method [13].

Real time polymerase chain reaction (RT-PCR) for *padi4* genotyping was performed by using specifically designed primers and probe. One set of primers was designed for *padi4* rs11203367 SNP (Forward: 5'- CCCTGGGGTAGAGGTGAC -3' and Reverse: 5'-

CTGAAGCCCATCCACACTG -3'). The utilized probe was FAM-BHQ Probe 5'-GAAAGTGGCCAGTGGTAGCA-3, VIC-BHQ probe 5'- ATGAAAGCGGCCAGTGGTAG-3. Primers and probe were custom-synthesized at Alpha DNA / Canada as a lyophilized product. DNase / RNase free water was used as a solvent for primers and probe to give a final concentration of 100 pmol /µl as a stock solution. Then, the working solution was prepared by adding 10 µl of the stock solution to 90 µl of nuclease-free water according to the formula: dH2O (C1V1=C2V2) and stored at -20 °C until use [14].

Real Time PCR reaction was performed in a total volume of 25µl, with 1µl of each of forward and reverse primers (20 pmol for each), 1 µl of each probe, and 4 µl template DNA (100 µg/ml). Forty RT-PCR cycles were carried out with the denaturation of the DNA template at 94^{o}C for 5 seconds and annealing at 60^{o}C for 30 seconds. An initial DNA denaturation at 94^{o}C was carried out for 1 minute.

Statistical analysis

Data analysis was achieved by utilizing SPSS for Windows, version 25 (SPSS Inc. Chicago, Illinois, United States). Allele and genotype frequencies were given as percentage frequencies. The genotype frequencies were first tested for their agreement with Hardy-Weinberg equilibrium (HWE) and significant differences between the observed and expected genotype frequencies were assessed by Pearson's Chi-square test. Tukey's, Dunnett, and Bonferroni Post Hoc tests for multiple comparisons were applied after ANOVA tests. The association between rs11203367 of *padi4* gene and RA was presented in terms of odds ratio (OR) and 95% confidence interval (CI). The *p*-value lower than 0.05 ($p<0.05$) was considered as significant.

Results

Agarose gel electrophoresis was used to confirm the presence and integrity of genomic DNA (Figure 1), followed by Nanodrop spectrophotometry to determine the purity and concentration of the extracted DNA.

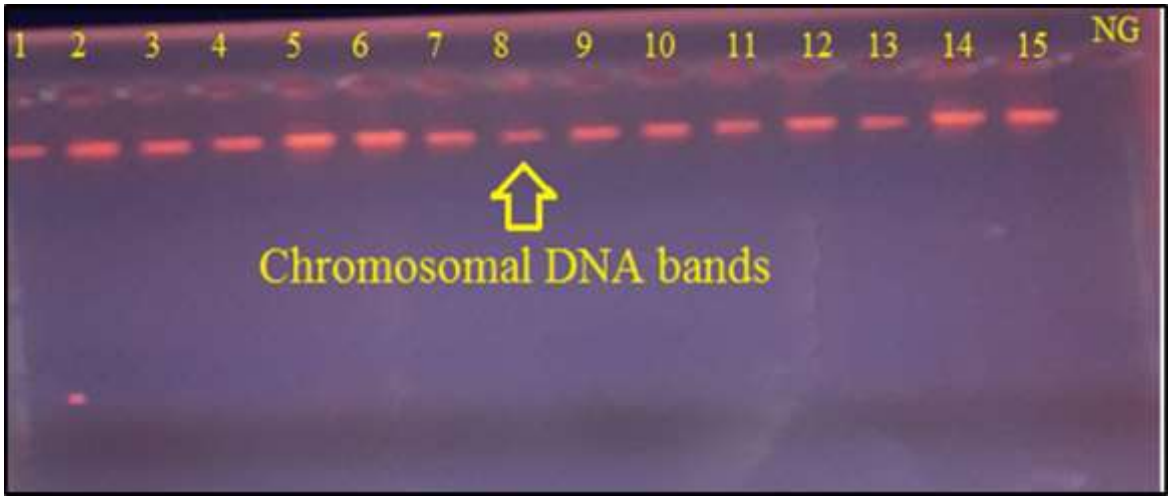

Figure 1-Chromosomal DNA bands extracted from human blood samples on 1.0 % agarose gel at 90 voltages for one hour, visualized under U.V light using gel documentation system after staining with Ethidium Bromide (EB) for half an hour. Lane (NG) is negative control while lanes (1-15) are of different samples.

Hardy-Weinberg equilibrium (HWE) analysis

The distribution of genotype frequencies in RA patients was in a good agreement with HWE, and no significant difference was noticed between the observed and expected genotype frequencies of the SNP rs11203367 (p = 0.751). Among control subjects, there was a significant difference from HWE (p = 0.041) (Table 1).

Table 1-Numbers and percentage frequencies (observed and expected) of *padi4* gene (rs11203367) genotypes and their Hardy-Weinberg equilibrium (HWE) analysis results in RA patients and controls.

Genotype	Rheumatoid arthritis patients (No. = 123)				Control (No. = 60)			
	Observed		Expected		Observed		Expected	
	No.	%	No.	%	No.	%	No.	%
TT	22	17.9	21.1	17.2	21	35.0	17.1	28.5
TC	58	47.1	59.7	48.5	22	36.7	29.8	49.7
CC	43	35.0	42.1	34.3	17	28.3	13.1	21.8
HWE Analysis	p = 0.751				p = **0.041**			

Genetic association of rs11203367 SNP with RA

By using RT-PCR, three genotypes of the SNP rs11203367 were detected; TT, TC and CC, which were correspondent to two alleles; *T* and *C* (Figure 2).

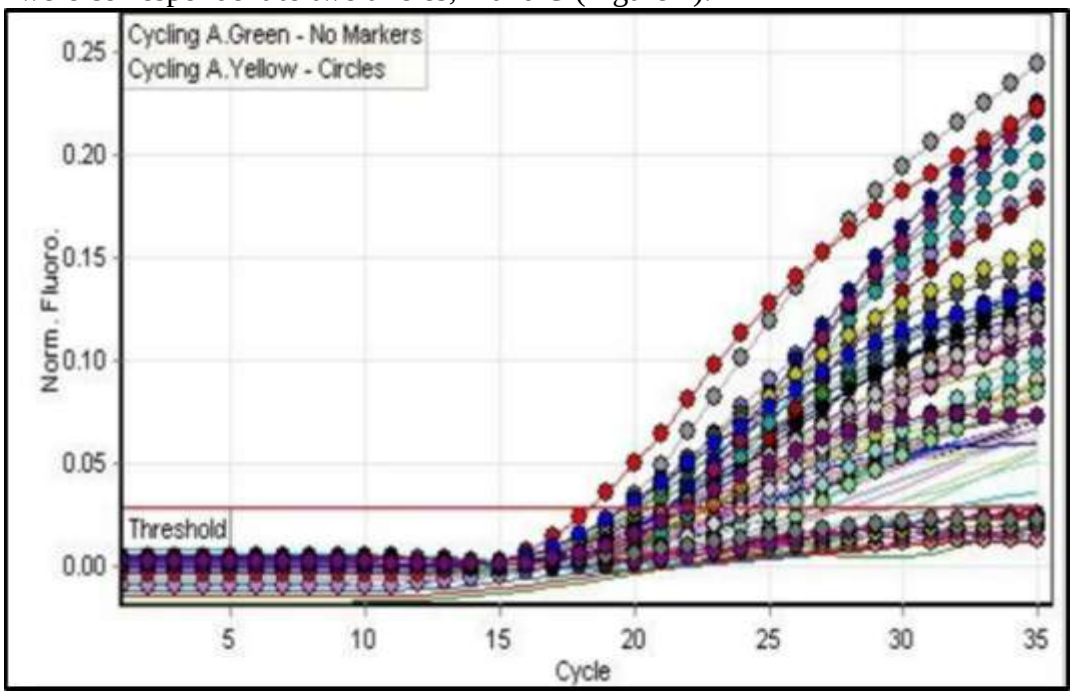

Figure 2-Typical Tacman-PCR for *padi4* gene rs11203367 showing allele discrimination.

Five genetic models were adopted to analyze the association between alleles and genotypes of rs11203367 and the risk of RA. Logistic regression analysis revealed some alleles and genotypes that were associated with susceptibility to RA in the present samples of Iraqi patients. Under the allelic model, *C* allele frequency was significantly increased in RA patients as compared to control (58.5 *vs*. 46.7%; p = 0.034). The OR of such difference was 1.61 (95% CI: 1.04 – 2.50). Under the dominant model, TC+CC genotype frequency was higher in patients than in control (82.1*vs*. 65.5%; OR = 2.47; 95% CI: 1.23 – 4.97; p = 0.015). Under the codominant model, frequency of the heterozygous genotype TC was significantly increased in patients as compared to control (47.1 *vs*. 36.7%; OR = 2.52; 95% CI: 1.17 – 5.41; p = 0.028) (Table 2).

Table 2-Genetic association analysis of rs11203367 SNP with RA.

Genetic model	Allele/ genotype	RA patients (No. = 123)		Control (No. = 60)		OR	95% CI	*p value*
		No.	%	No.	%			
Allele	*T*	102	41.5	64	53.3	Reference		
	C	144	58.5	56	46.7	1.61	1.04 - 2.50	**0.034**
Recessive	TT+TC	80	65.0	43	71.7	Reference		
	CC	43	35.0	17	28.3	1.36	0.70 - 2.65	0.405
Dominant	TT	22	17.9	21	35.0	Reference		
	TC+CC	101	82.1	39	65.0	2.47	1.23 - 4.97	**0.015**
Overdominant	TT+TC	65	52.8	38	63.3	Reference		
	TC	58	47.2	22	36.7	1.54	0.82 - 2.89	0.206
Codominant	TT	22	17.9	21	35.0	Reference		
	TC	58	47.1	22	36.7	2.52	1.17 - 5.41	**0.028**
	CC	43	35.0	17	28.3			

OR: Odds ratio; CI: Confidence interval; *p*: Two-tailed Fisher's exact probability.

Discussion

Rheumatoid arthritis is a complex disease where genetic factors play a key role in susceptibility. The *padi4* gene is one of the most important RA susceptibility loci in multiple ethnic groups. In the beginning, *padi4* was identified as a non-MHC RA risk locus in Asian populations, especially in Japanese, followed by its recognition in European and Latin American populations [15, 16]. In Asian populations, the padi4 gene seems to have a significant association with RA disease, but in Caucasian populations, such association is not confirmed. These populations vary in their environmental variables, which may play a role in the previous findings [17].

The results of the current study are consistent with the findings in the Korean [18], German [19], French [20], European [21] and Mexican populations [22-24]. On the other hand, the results of this study are inconsistent with that of Chen *et al.* in Chinese population [25] and Shamsian *et al.* in Iranian population [26]. Genetic associations are not the only factors, because the disease is also affected by the differences in the ethnic groups and genetic background, along with the clinical aspects, such as disease severity [23]. This meta-analysis demonstrated that *padi4* polymorphism represents a significant risk factor for RA disease.

A study by Harris *et al*. mentioned three non-synonymous polymorphisms (rs11203366, rs11203367, and rs874881) associated with RA disease susceptibility [27].]. In a meta-analysis of 1019 RA patients and 907 controls according to sex, the *padi4* gene polymorphism (rs11203367) was significantly associated with the disease only in males [28]. The important association between rs11203367 and the GTG haplotype with a functional disability was shown to have a slight tendency [23]. There are major variations in the association of *padi4* SNPs with RA in the Indian population, which may be due to differences in ethnicity [29].

In other studies for other diseases, such as the research by Chen *et al.*, no significant differences were found in *padi4* rs11203367 allele frequency between patients with Ankylosing Spondylitis (AS) and controls in Chinese Han population [30]. Chenou *et al.* reported no association between the *padi4* rs11203367 polymorphism and the history of leg ulcers (LU), Vaso-Occlusive Crisis (VOC) and stroke [31]. Another research by Massarenti *et al.* in Iranian population demonstrated that alleles of five SNPs (rs874881, rs11203366, rs2240340, rs11203367, and rs11203368) were associated with increased occurrence of lupus nephritis (LN) and hypertension [32].

Other SNPs on *padi4* gene are associated with RA disease in Japanese population [33, 34]. Genetic heterogeneity may have a role in the varied association of *padi4* with RA between the populations in Europe and Asia [18]. In Chinese population, Fan *et al.* examined the

distribution of four exonic SNPs of the *padi4* gene (rs11203366, rs11203367, rs874881, rs1748033). The authors stated that *padi4* expression was significantly higher in RA patients, with this increase being correlated with the alleles of the four SNPs [35].

Conclusions

The major findings of the current examination of allele distribution of rs11203367 of *padi4* gene revealed that *C* allele represents a risk allele. In conclusion, our results provide an evidence of a clearly significant correlation between rs11203367of *padi4* gene and RA in the Iraqi population.

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