

Effects of Genetic Polymorphisms of Programmed Cell Death-1 in Susceptibility with Rheumatoid Arthritis in Najaf Population

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ABSTRACT

Background: The prevalence of Rheumatoid Arthritis (RA) has been raised in Iraq. The medical costs of such increased prevalence are high. Global studies have revealed polymorphisms of *PDI* (Program Death 1) gene to be associated with Rheumatoid Arthritis (RA). This polymorphism can cause changes in various metabolic variables.

Methods: The current study consists of 100 RA patients and 100 healthy control individuals. Variable parameters data included Anti-Cyclic cytrulinated peptide expression (ACCP), Rheumatoid factor (RF), C-Reactive Protein (CRP) in serum and Erythrocytes sedimentation rate (ESR) levels. Genotyping of *PDI* gene polymorphism is carried out by RFLP-PCR. Various statistical analyses were applied to analyze the data.

Results: The estimation of immunological and biochemical data pointed out significant differences in Gender, RF, ACCP, CRP, ESR and age in RA when compared with those of the control group. The genotyping results were found to be consistent with Hardy-Weinberg equilibrium. The analysis of the genotype distribution under various inheritance models highlighted significant differences in SNP of *PDI* gene polymorphism among RA patients when compared with the control group under the dominant homozygote, heterozygote and recessive homozygote. The genotype and allele frequencies of the *PDI* SNP rs11568821; genotype GA was significantly increased in RA compared with controls (45% and 24%, respectively) ($P=0.0020$), ($OR = 2.5909$), (95% CI 1.4-4.7). Subjects who carried A allele were significantly more likely to develop RA three folds AA ($OR=3.3529$, 95% CI=1.1691-9.6160, $P=0.0244$). Clinical characteristics were observed to change significantly with respect to the genotype distribution of the investigated SNP. **Conclusion:** *PDI* gene polymorphisms (rs11568821) is implicated in the pathogenesis of Rheumatoid arthritis in Najaf population.

Keywords: Rheumatoid arthritis, *PDI*, rs11568821, RFLP-PCR, ACCP, CRP, RF.

INTRODUCTION

Rheumatoid arthritis (RA) is a common "chronic, autoimmune and systemic inflammatory" joint "disease". It affects about 1% of the world people; however, prevalence differs between 3 and 8% depending on the inherited and environmental risk factors (Prasannavar et al, 2014). Even with these declines, "RA occurs at twice the rate in women if we compare with men", with a prevalence of 1.06% in women as a percentage of the total population compared with 0.61% in men (Gibofsky, 2012).. Although the RA can occur at any age of human, its incidence increases with age and most cases have an onset between 40 and 70 years (Thyberg et al, 2009). The disease being the common inflammatory affects multiple joints causing "poly-arthritis". This condition, if not cured at earlier stages, results in major joint disability and impairment (Gibofsky, 2012; Scott et al, 2010; Wang et al, 2014). The diagnosis of disease especially in the early time is quite impossible, as the clinical criteria are

insufficient at the early stage of the RA. In last 5-6 years, many researches have focused on the value of the diagnostic probability and clinical application of anti-CCP antibody in RA and other rheumatic diseases (Chou et al, 2007). RA is characterized by a proliferative disorder of synovial tissue associated with Th1-predominant immune dysregulation (Dolhain et al, 1996). RA pathogenesis is the come from a "complex interaction between genetic and environmental factors", auto-antigen presence with "antigen specific" T and B cells activation and aberrant inflammatory cytokines production (Fedetz, 2009). Bartok, et al confirm that much of the effort to define the RA epigenome has focused on fibroblast-like synoviocytes (FLS) of the synovial intimal lining, which attack the cartilage and assume a unique aggressive phenotype in patients with Rheumatoid arthritis (Bartok and Firestein, 2010). Programmed cell death-1 (*PDI*) gene is a negative regulator of T-cell to maintain peripheral tolerance and is a key molecule in the development of autoimmune diseases. Although gene polymorphism in *PDI* was reported to be associated with rheumatoid arthritis (RA), replication studies later on showed conflicting results. This study aimed to determine whether SNP PD1.3 in *PDI* gene is associated with susceptibility for RA (Jawad et al,2017).

METHODS

The study has been carried out on 100 Rheumatoid arthritis patients (36 male and 64 female). The ages of patients ranged between 15-75 year with a mean \pm SD of 44.4300 ± 14.4182 year. They were selected from AL-Sader Teaching Hospital, Al-Hakeem Hospital and AlForat Al-Awsat Hospital in al Najaf. Diagnosis of RA was confirmed by specialist physicians for the inclusion of the patients. The control group consisted of 100 obviously healthy subjects (40 male and 60 female). The ages of the control individuals ranged between 19-76 year with a mean \pm SD of 42.630 ± 13.8277 year. The collection of samples is done from April 2018 till January 2019. The biochemical methods, immunological methods and genetic methods were carried out in the laboratories of Biology Department of the Faculty of Science/University of Kufa. All patients were fulfilled the ACR/EULAR 2010 criteria for the classification of RA (Aletaha et al, 2010), as shown in table (1). The biochemical parameters were accomplished including estimation sedimentation rate ESR, C-Reactive protein CRP, Rheumatoid Factor RF, Anti-cyclic citrulinated protein ACCP, see table (2). DNA has been extracted from blood using DNA purification kit (Geneaid). Genotyping has been carried out by using PCR-restriction fragment length polymorphism (RFLP) for gene of *PDI*. Amplification for SNP was carried out with the use of suitable primers and a 2X Master Mix with standard Buffer kit (Biolabs). Products of PCR have been digested with restriction enzymes *PstI* (Takara, Japan). The digested products were separated on a 2.2% and 2.5% agarose gel.

Table (1) Characteristics of study subjects

Parameter	Control subject	RA subject	P-value
No. (M/F)	100(40/60)	100 (36/64)	P> 0.05"
Age (year)	42.630 \pm 13.8277	44.4300 \pm 14.4182	P = 0.0181"
ESR (mm/h)	10.7300 \pm 3.9667	55.1900 \pm 20.3478	P < 0.0001"

Table (2) Characteristics RF, ACCP and CRP of study subjects for patients RA

Parameter	RA subject n(100)	P-value	Comparing With Control
Positive(%)			
RF	76 (76%)	24(24%)	P<0.05
ACCP	81(81%)	19(19%)	P<0.05
CRP	67(67%)	33(33%)	P<0.05

EXTRACTION OF DNA, DETERMINATION OF CONCENTRATION AND PURITY The extraction containing 2ml of blood put in EDTA tube for genotype analysis. From patient and control subjects, collected peripheral blood samples in EDTA tubes were subjected to DNA extraction from whole blood specimens applying gDNA Miniprep System of gSYNC™DNA Extraction Kit (100 preps)(Geneaid). The concentration and purity of DNA were assessed by measurement of the A260/A280 ratio. Purity of DNA samples (Assessment of Nucleic Acid Purity)was established and the range of concentration of the extracted DNA was 32-240µg/ml of patient groups. Results were clarified in table (3). Quantified DNA samples were stored at -20°C until used for genetic analysis. **Table (3): Concentration and purity of DNA of patient groups**

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DNA	Mean ±SD
Concentration of DNA (µg/ml)	92.35±12.65
Purity of DNA	1.85±0.04

AMPLIFICATION REACTIONS RESULTS The product of *PDI* gene polymorphism analyzed for amplicon size for rs11568821was 180 bp. Result of amplification of gene polymorphism was analyzed and confirmed by electrophoresis on agarose gel as illustrated in figure (1).

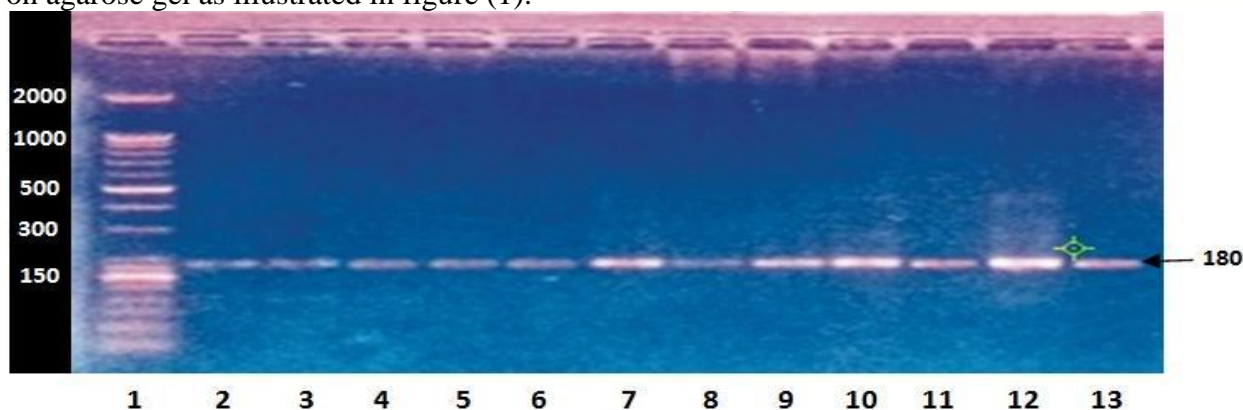


Figure (1): PCR product of rs11568821 SNP of *PDI* gene polymorphism analyzed by agarose gel electrophoresis. Lane 1:marker of DNA (25bp –2kb).Lanes 2-13: Amplicon size of PCR product, 180 bp.

DETECTION OF *PDI* POLYMORPHISM

The *PDI* polymorphism that located in Intron 4 (+ 7146 G>A) was genotyped by PCR-RFLP method. was amplified by PCR from genomic DNA followed by digestion with restriction enzyme *PstI* (Takara, Japan), The primer sequences employed to amplify Program Death1 (*PDI*) gene analyzed for polymorphisms (rs11568821) as follows, 5'CCCCAGGCAGCAA CTCAAT-3' (Forward) and 5'GACCGCAGGCAGGCACATAT-3' (Reverse) IDT Integrated DNA Technologies (HK). One *Taq*® Quick-Load® 2X Master Mix with Standard Buffer is a premixed ready-to-use solution containing *Taq* Polymerase, dNTPs, MgCl₂ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Briefly, each 25µl PCR reaction contained One *Taq*® Quick-Load® 2X Master Mix with Standard Buffer 12.5 µl, Forward-primer 1.5 µl, Reverse-primer 1.5 µl, Genomic DNA 7 µl and Nuclease free water 2.5 µl. Reactions were carried out in a gradient thermocycler (Agilent Technologies, USA) under the following conditions; initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 40sec, annealing at 61°C for 50sec, extension at 72°C for 40sec and final extension cycle at 72°C for 6 min. The product of *PDI* 180 bp was then digested with *PstI* restriction enzyme under the following conditions; 15µl of the reaction were incubated with 1.5U of *PstI* (Takara, Japan) at 37°C for overnight along with its corresponding buffer. Digested products were electrophoresed on a 2.5% agarose gel in TBE buffer along with 25bp ladder. Products of digestion: not cutting Allele G:180, the cutting existing with Allele A: 123 bp and 57 bp, finally heterozygous GA genotype: 180 bp, 123 bp and 57 bp. as shown in figure (2).

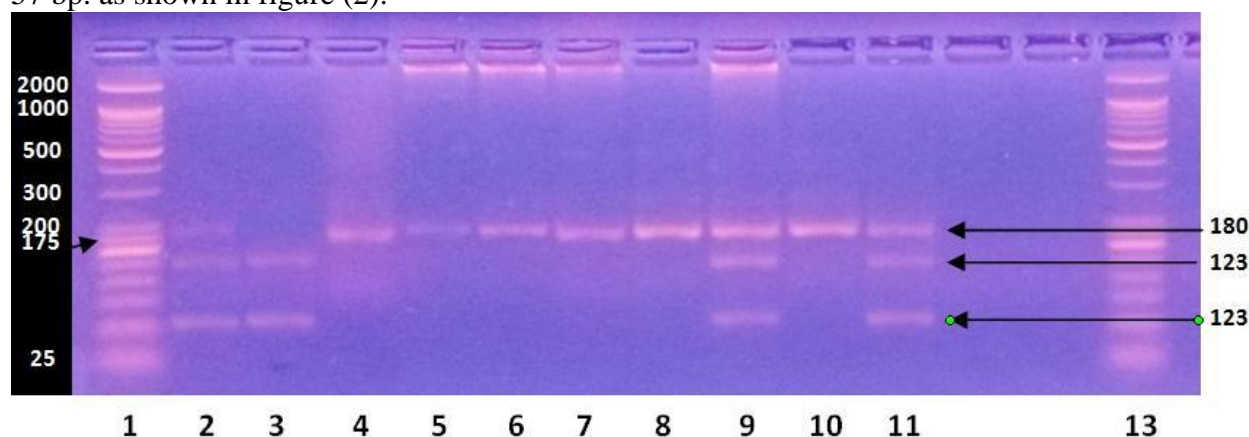


Figure (2) PCR product of rs11568821 G↔A SNP of *PDI* gene digested by restriction enzyme and electroporesed on 2.5% agarosegel electrophoresis. Lane 1 and 13: Marker of DNA 25 bp. Lanes 4, 5, 6, 7, 8 and 10 :GG genotypes without digestion 180 bp. Lane 2,9 and 11: GA genotype 180, 123, 57bp. Lane 3: AA genotype123,57 bp.

STATISTICAL ANALYSIS

The mean ±SD term is used to express the continuous variables. The differences in means between RA Patients and group of control were determined by Student's t-test. By using MedCalc software for comparing level means of continuous parameters across genotypes and multi nominal logistic regression analysis was achieved to assess the association of genotype and allele frequencies with RA under different inheritance models. The ANOVA test is applied to determine the relation between genotype distribution and bio-parameters. Genotype distribution and allele frequency

are expressed as non- numerical variables. Differences of these variables are assessed by the chi-squared test. Changes are considered significant when the p value was <0.05. The mathematical relationship that relates genotypes to allele frequencies is the Hardy–Weinberg equilibrium (HWE) test (Thompson *et al*, 1991).

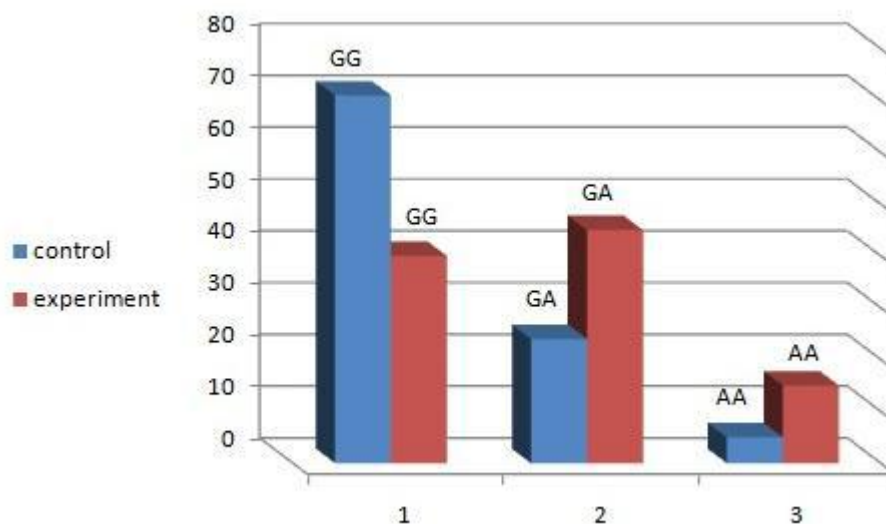
RESULTS

The frequencies of *PDI* (rs11568821) GA genotype were significantly increased in RA compared with controls (45% and 24%, respectively) ($P=0.0020$), (OR =2.5909), (95% CI 1.4-4.7). Subjects who carried A allele were significantly more likely to develop RA three folds AA (OR=3.3529, 95% CI=1.1691-9.6160, $P=0.0244$). There was no significant difference between patients with RA regarding *PDI* genotypes GG, as described in Table (4). The distribution of allelic frequencies, as shown in figure (3).

Table (4) Frequency estimation of genotype and allele for *PDI* gene polymorphism (rs11568821) in RA and control subjects

Alleles (genotypes)	RA patients (n=100)	Control (n=100)	Total (n=200)	Odds Ratio	95%CI	(p-value)
GG Wild type	40(40%)	71(71%)	111	0.2723	0.1511-0.4906	$P < 0.0001^*$
GA	45(45%)	24(24%)	69	2.5909	1.4–4.7	$P = 0.0020^*$
AA	15(15%)	5(5%)	20	3.3529	1.1691-9.6160	$P = 0.0244^*$
G	62(62%)	85(85%)	147	1	-	-
A	38(38%)	15(15%)	53	2.4	1.7–2.5	$(0.0001)^*$

* $P < 0.05$, significant.



Figure(3) Distribution of the *PDI* gene rs11568821 SNP genotypes among RA cases compared with healthy control . In each graph columns show wild type GG, heterozygote GA, homozygote AA and total mutant allele frequencies respectively

Biochemical parameters erythrocyte sedimentation rate (ESR) as well as serological parameters; C-Reactive Protein (CRP), Rheumatoid Factor (RF) and Anti-Cyclic

Citrullinated Protein (ACCP) values were analyzed in relevance to the genotype of the studied SNP of *PD1* gene in RA patients under inheritance models by ANOVA test. The results according to the rs11568821 SNPs of *PD1* gene RA patients, all of them demonstrated that existed significant difference in ESR (P=0.001), CRP (P=0.01) and ACCP (P=0.005) except RF (Rheumatoid factor) no statistical difference is present.

The following table (5).

Table (5) Association of Clinical characteristics with *PD1* gene polymorphism of RA patients.

characteristic	GG (n=40)	GA (n=45)	AA (n=15)	P- value
ESR (mm/h)	46.62±56.4	48.43±39.9	66.28±48.1	0.001*
CRP+ (n = 67)	21(52.5%)	32(71%)	14(93.3%)	0.01*
RF+ (n = 76)	28 (70%)	35 (77.7%)	13 (86.6%)	0.439
ACCP+ (n = 74)	21 (52.5%)	40 (88.8%)	13 (86.6%)	0.005*

*P<0.05, significant.

DISCUSSION

PD1 is an immune transmembrane receptor tyrosine based on inhibitory molecule expressed on lymphocytes in the B, activated T cells and thymus (Prokunina et al, 2004). *PD1* gene plays an important role in regulation of the induction and maintenance of T-cell tolerance, and it inhibits the effector T-cell responses that protect tissues from autoimmune-mediated tissue damage (Dai et al, 2014). Experimentally, the mice lacking *PD1* gene developed autoimmune diseases, indicating the essential function of *PD1* in the mechanisms of immune tolerance (Zhang et al, 2014). Bearing in mind the fact that mutant allele A of SNPs is located in promoter region of *PD1* gene, it can probably influence the gene expression of it; so, the current research was designed to study such an association of existing this allele with not existing .

In the present study, there are no significant differences between the age, sex and variant of allelic distributions, *PD1* (rs11568821) "gene polymorphism represented that GA and AA genotypes were significantly higher among patients with RA". "On the other side, GG genotype was significantly less frequent". Thus, there was increased frequency of A carrier and A allele in patients with RA. There are many agreements with us about the same findings of present study ; in Sweden and Denmark population studies were performed by Prokunina et al (2002, 2004). The mutant A allele in rs11568821 (*PD-1* G/A) polymorphism represents a higher risk of RA in Caucasians (Zou et al, 2017), in Southern Brazilian population (do Canto et al, 2016), Ibrahim et al suggests relation between the PD1 SNP and Rheumatoid arthritis in Egyptian people (Ibrahim and Abdelghani, 2018), study on South-Eastern Poland by (Siwiec et al, 2015), Tseng et al investigate and suggest a relation in Taiwanese cohort (Tseng et al, 2019). Clinical characteristics of study subjects with respect to genotyping, The previous table explains a statistical significant difference between RA patients each other according to have the risk factor of disease (A allele), either GA or AA in genotype of patients. ESR; there is significant difference (P=0.001) for GG slightly decrease value (46.62±56.4) compared with GA (48.43±39.9) and AA (66.28±48.1). Then, there is a difference according to CRP (P = 0.01) our findings refer to decrease in CRP of GG genotype (52.5%), in contrary elevating in GA (71%) and finally AA (93.3%).

All these results in ESR, CRP and ACCP values suggested the participation of the rs11568821 SNP Intron 4 (+ 7146) of *PD1* gene in directing metabolic changes of Rheumatoid arthritis.

CONCLUSION *PD1* gene polymorphism (rs11568821) is implicated in the pathogenesis of Rheumatoid arthritis in Najaf population. From other side, the metabolic changes; including ESR, C-RP, RF and ACCP levels and counts are relevant to this gene.

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