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Association of Polymorphisms in the NLRP3 Gene and Rheumatoid Arthritis in Iranian Patients

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a complex systemic autoimmune disorder with multifactorial nature. Numerous previous studies have shown that several genes are involved in the pathogenesis and increased risk of RA. The Nod-like receptor pyrin domain containing 3 (*NLRP3*) is involved in the regulation of innate immunity and its upregulation has previously been reported in RA.

Objective: To evaluate the correlation between 3 functional polymorphisms of NLRP3 and its gene expression and RA risk.

Methods: One hundred and fourteen patients with RA and 120 healthy participants were recruited to this case-control study. Genotyping of rs4612666 (intronic variant), rs10754558 (3UTR variant), and rs6672995 (downstream variant) were performed applying the realtime polymerase chain reaction highresolution melting (HRM) method.

Results: Based on logistic regression analysis, subjects with CC genotype and C allele in rs4612666 had increased risk of RA (OR $_{\text{for CC genotype}}$=3.10; 95%CI [1.78-8.26]/ OR $_{\text{for C allele}}$=2.00; 95%CI [1.45-3.10]). Furthermore, in the patient groups, there was a significant relationship between the concentration of C-reactive protein (CRP) and rs4612666 and rs10754558 polymorphism (P<0.05). Besides, our results revealed no significant association between the genotype and allele frequency of rs10754558 and rs6672995 and the risk of RA (P>0.05).

Conclusion: Our findings propose a significant association between rs4612666 polymorphism and increased risk of RA in the Iranian population. Moreover, rs4612666 and rs10754558 were correlated with disease activity.

Keywords: Arthritis, Genotypes, Inflammasome, NLRP3 Gene, Rheumatoid, Single Nucleotide Polymorphism

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INTRODUCTION

Rheumatoid arthritis (RA) is a complex systemic multifactorial autoimmune disorder , known as one of the most common types of inflammatory arthritis (1). The RA disease is described by long-term inflammation in the joints that finally results in deformities and bone erosion (1-3). Regarding the chronic and systemic inflammatory nature of this disease, patients with the RA disease have an increased risk of cardiovascular disorders (4). The prevalence of the RA is about 1% and generally emerges approximately at the age of 35 (5, 6). There are established links between many environmental and genetic factors to an improved risk of the RA that mirrors the multifactorial etiology of the RA (3, 7). Previous twin studies have estimated the heritability of the RA at around 50–60% (8). Although several genes play a role in the occurrence of the RA, at present, these genetic factors just explain only 50% of the RA heritability (9, 10). Single nucleotide polymorphisms (SNPs), the common form of allelic variations in the DNA sequence, occur, on average, once every 300 nucleotides with the frequency ≥ of 1% for minor allele and might be related to diseases, particularly multifactorial disease including the RA (11-13). Currently, with progress in genotyping technology, the SNP-based association studies discovered over one hundred susceptibility loci related to the RA in different populations (14-16). Numerous studies reported that several SNPs in genes involved in the immune system especially inflammation are associated with the RA disease by modulating their function (17, 18). Nod-like receptor pyrin domain containing 3 (*NLRP3*) is one of these genetic factors that expresses an intracellular receptor leading to the activation of the NLRP3 inflammasome after distinguishing endogenous pathogens signals (19, 20). The formation of the NLRP3 inflammasome caused the release of the potent proinflammatory cytokines and, in turn, these factors elicit the production and release of other inflammatory cytokines for instance IL-18 induces the excretion of TNFα, IL-1β, and IL-8 (19, 21). Therefore, overactivation of NLRP3 inflammasome results in excessive inflammation and subsequently unnecessary host tissue damage (22). Accumulating evidence proposes that the NLRP3 is critically associated with the initiation and progression of autoimmune disease; the expression of NLRP3 is upregulated in various immune cells in individuals with systemic lupus erythematosus (SLE) (16, 23-25), systemic sclerosis (SSc) (26), and especially in the RA disease (27-30). Furthermore, previous researches have established that NLRP3 polymorphisms are correlated to the modification of disease susceptibility, severity, and response to treatment of this type of disorder (25, 31-33). Previous studies have demonstrated that in the NLRP3 gene there is some functional polymorphism such as rs4612666 (intronic variant), rs10754558 (3UTR variant), and rs6672995 (downstream variant) which potentially could lead to up or down-regulate the expression of the gene and finally downstream genes such as IL-1β; this process consequently could change autoimmune diseases susceptibility (34-38). To our knowledge, this is the first time we analyzed the possible link between these variants in the current project in the NLRP3 gene with the RA susceptibility in Iranian patients. Similarly, the interaction between these variations and a number of laboratory parameters was investigated in order to determine their impact on adjustment of the RA risk and disease activity. There was a substantial connection on the basis of our findings between rs4612666 polymorphism and increased risk of RA. Furthermore, rs4612666 and rs10754558 in the NLRP3 gene were connected with disease activity in the population under study.

MATERIALS AND METHODS

Subjects Population

This case-control study registered 114

RA patients according to diagnostic criteria of the American College of Rheumatology (ACR), (2010) and 120 age and sex-matched healthy participants in the control group. None of the healthy controls had any history of all autoimmune diseases or other immune-related diseases. The individuals were asked to fill out a questionnaire to acquire data on the known elements to induce the RA risk such as age, gender, blood pressure, family history of the RA disease and similar disorders. Similarly, we listed laboratory characteristics including serum concentration of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cell (WBC) count, hemoglobin, creatinine, triglyceride, and blood urea nitrogen (BUN). The other laboratory and demographic factors are listed in Tables 1 and 2. This study was authorized by the AJA University Research Ethics Committee (UREC), and the participants signed informed consent forms. Finally, approximately 3cc of peripheral blood were collected into EDTA anticoagulant tubes from the subject and maintained at -20°C until DNA isolation.

SNP Selection and Genotyping

We identified three variations that impact the transcription of the NLRP3 gene after conducting a comprehensive literature research. These variants include

Table 1. Baseline characteristics of RA patients and control subjects participating in this study

Characteristics	Patients	Controls	P value
Total number	114	120	
Age at sampling time	47.4±10.49	45.39±12.73	0.189
Gender n (%)			
Male	32(28.1%)	39(32.5%)	0.480
Female	82(71.9%)	81(67.5%)	
Age of onset	41.13±10.44	--	--
BMI	26.22±2.47	24.14±3.31	<0.001*
SBP	122.46±12.50	120.92±9.74	0.296
DBP	78.42±7.80	78.75±8.28	0.755
Positive family history n (%)	20(17.5)	0	<0.001*

*P value<0.05. RA: Rheumatoid arthritis; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure

Table 2. Laboratory characteristics of patients with RA and control groups

	Patients (114)	Controls (120)	P value
ESR (mm/h)	36.65±23.20	15.57±6.92	<0.001*
CRP (mg/l)	16.41±18.61	4.45±2.58	<0.001*
White blood cell (10^9/l)	7.33±2.18	6.57±1.37	0.002*
Hemoglobin (HB)	12.43±1.07	14.33±1.59	<0.001*
PLT (10^9/l)	261.74±58.17	251.02±66.77	0.239
Creatinine (mg/dL)	1.03±0.18	0.86±0.18	<0.001*
BUN	17.15±4.68	16.11±4.09	0.073
FBS	96.46±15.84	92.92±21.95	0.157
HDL	49.35±7.63	50.41±11.07	0.394
LDL	110.06±29.08	107.03±31.29	0.445
TG	169.32±48.32	155.58±59.86	0.055

*P value<0.05. RA=Rheumatoid arthritis; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; BUN: Blood urea nitrogen; PLT: Platelet; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglyceride; FBS: Fasting blood sugar; SD: Standard deviation

Table 3. Primer sequences for the amplification of fragments around the three polymorphisms of the NLRP3 gene.

SNP ID	Primer sequence	PCR product length (bp)	Annealing temperature
rs4612666	F: CCACAATAAAGCTGAATGTAGGGAG R: CACATGGAAAGGGAGTGGACA	138	59°C
rs10754558	F: CAGGGTGAGGAAGACACCAG R: GAGCTAATTACATGAGGTCACCA	103	60°C
rs6672995	F: AATGGTAAGGTCCCAGCAGC R: GGGTTCCTGGCTCCTACAGA	169	59°C

rs4612666 located in an intronic regulatory sequence (intron 7) and rs6672995 situated in the downstream regulatory site and might modulate the expression of NLRP3 (35, 36, 38). A subsequent variant is rs10754558 located in the 3UTR portion of the gene, involved in changing mRNA stability by an effect on the secondary structure of NLRP3 mRNA and alter the capacity of miRNA-mRNA binding events (35, 37).

Isolation of human genomic DNA performed by appropriate DNA isolation kit mentioned in our previous studies (39, 40). The quantity, purity, and propriety of DNA for genotyping were measured with the spectrophotometry method. The forward and reverse sequence-specific primers used for amplification of fragments around these three variants in the NLRP3 gene are listed in Table 3. Our detailed methodology for genotyping including the type of HRM kit and temperature program were previously described (16).

Statistical Analyses

We utilized the SPSS 25 (Armonk, NY: IBM Corp) for statistical examination. Hardy-Weinberg equilibrium (HWE) was confirmed between two groups for genotype frequencies via the chi-square (χ^2) statistic. Logistic regression was performed to examine the relationship among genotypes and the RA and to estimate p-values, odds ratios (ORs), and 95% confidential intervals (CIs). For other characteristics such as clinical features, p-values were considered by Student's t-test or the chi-square (χ^2) test.

RESULTS

Demographic and Laboratory Features

To evaluate the correlation among polymorphisms with the occurrence of RA, 234 participants in the patients and the control groups were analyzed; 114 patients (82 females and 32 males) with the mean age of 47.4±10.49 in the cases and one hundred twenty volunteers (81 females and 39 males) with a mean age of 45.39±12.73 in the healthy subjects group. The mean age of onset in the RA patients was 44.20±10.63 years. No considerable relationship between the two groups considering sex (P=0.480) and age (P=0.189) was seen, an index that for these variables matching was acceptable. The distributions of selected parameters of the two groups are documented in Table 1. Among the two groups of participants, a noteworthy variance in terms of body mass index (BMI) and positive family history of the RA and other similar autoimmune diseases was divulged (P<0.001). No variance between the case and control group in blood pressure parameters was uncovered (Table 3; P>0.05). According to laboratory tests, the serum level of ESR, CRP, and creatinine was significantly greater in the cases than in the non-RA subjects (P<0.001). Similarly, the WBC count in the cases was superior compared with the healthy volunteer group (P=0.002). Instead, the level of hemoglobin was meaningfully lesser in the RA case subjects compared with the control subjects (P<0.001). Other laboratory elements such as BUN, HDL, LDL, TG, FBS, and PLT were

not distinct between the case and the control participants (P>0.05). The detailed laboratory parameters of the cases and the controls are documented in Table 1.

Allele Frequency and Genotype Distribution of rs4612666

The distribution of different genotypes at rs4612666 (C>T) variant in the patients and the healthy individuals were in corroboration with HWE. The frequencies of TT, TC, CC genotypes in the case group were 11.4%, 37.7%, and 50.9%, respectively, whereas the distribution of genotypes in the non-RA group was 25.8%, 44.2%, and 30%, respectively. Significant association was found between CC genotypes (compared with TT; (P=0.001)) and the RA risk. Comparing the combined genotype, our data established that the TC+CC (88.6% in patients compared with 74.2% in the controls) compared to the TT (11.4% in the cases compared with 25.8% in the controls) genotype increases the risk of the RA susceptibility (P=0.007). In addition, the percent of subjects with T and C alleles were 47.92% and 52.08% in the non-RA group, and 30.3% and 69.7% in the RA patients, respectively, and C allele was connected with an augmented incidence of the RA (P<0.001) (Table 4). Besides, our stratification analysis revealed that there was no important correlation between age of

Table 4. Association between genotypes and allele frequency of NLRP3 polymorphisms with RA risk

Genotype group	Patients (n=114) n (%)	Controls (n=120) n (%)	OR (95%CI)	P value
rs4612666				
TT	13 (11.4%)	31 (25.8%)	Reference	---
TC	43 (37.7%)	53 (44.2%)	1.60(0.90-4.15)	0.063
CC	58 (50.9%)	36(30%)	3.10(1.78-8.26)	0.001*
Combined Genotype				
TT	13 (11.4%)	31 (25.8%)	Reference	---
TC+CC	101(88.6%)	89 (74.2%)	2.13(1.33-5.50)	0.007*
Allele				
T	69 (30.3%)	115 (47.92%)	Reference	---
C	159 (69.7%)	125 (52.08%)	2.00(1.45-3.10)	<0.001*
rs10754558				
CC	50 (43.9%)	47 (39.2%)	Reference	---
GC	40 (35.1%)	44 (36.7%)	1.17(0.48-1.53)	0.598
GG	24 (21.1%)	29 (24.2%)	1.10 (0.4-1.52)	0.790
Combined Genotype				
CC	50 (43.9%)	47 (39.2%)	Reference	---
GC+GG	64(56.1%)	73 (60.8%)	1.21(0.50-1.39)	0.508
Allele				
C	140 (61.4%)	138 (57.5%)	Reference	---
G	88 (38.6%)	102 (42.5%)	1.18(0.59-1.23)	0.390
rs6672995				
GG	82 (71.9%)	92 (76.7%)	Reference	---
AG	18 (15.8%)	21 (17.5%)	1.01(0.48-1.93)	0.969
AA	14 (12.3%)	7 (5.8%)	2.33 (0.86-5.81)	0.129
Combined Genotype				
GG	82 (71.9%)	92 (76.7%)	Reference	---
AG+AA	32(28.1%)	28 (23.3%)	1.28(0.71-2.31)	0.407
Allele				
G	182 (79.8%)	205 (85.4%)	Reference	---
A	46 (20.2%)	35 (14.6%)	1.48(0.91-2.40)	0.110

*P value<0.05

onset, sex, ESR, creatinine, and hemoglobin subgroups with SNP genotypes in patients (P>0.05). While the mean serum level of CRP in the RA subjects was meaningfully dissimilar in different genotypes (P<0.001). In detail, individuals in the case group with the C allele have an advanced level of CRP (Table 5).

Allele Frequency and Genotype Distribution of rs10754558

By comparing allele and genotype frequencies of the rs10754558 (G>C) variant among the RA patients and the healthy individuals in the control groups, we have unveiled no noteworthy differences (P>0.05). The frequency of the rs10754558 genotypes, CC, GC, and GG were 39.2%, 36.7%, and 24.2% in the control groups and 43.9%, 35.1%, and 21.1% in the case groups, respectively. When we compared combined genotype GC+GG/CC as variant genotype, the GC+GG genotype had no augmented or reduced risk in association with the RA (P=0.508). Additionally, the percent of participants with C and the G alleles were 57.5% and 42.5% in the healthy individuals, and 61.4% and 38.6% in the patients, respectively (Table 4). Likewise, the serum level of CRP in the RA group is considerably diverse in cases with different genotypes (P<0.001). In detail, the CRP level in cases with CC, GC, and GG genotypes were 8.77±7.58, 12.76±7.00, and 38.40±29.02, respectively, which means the RA individuals with the G allele have a greater serum level for CRP. While other stratification analyses

Table 5. Association of NLRP3 polymorphisms with various parameters of RA disease

	rs4612666			P value
	TT (n=21)	**TC (n=43)**	**CC (n=50)**	
Age of onset	44.08±12.59	38.65±10.17	42.31±9.92	0.122
		Sex		
Males	1(4.8 %)	13(30.2%)	18(36.0%)	0.220
Females	20(95.2%)	30(69.8%)	32(64.0%)	
ESR (mm/h)	23.39±7.63	36.33±21.15	39.86±26.01	0.067
CRP (mg/l)	4.32±2.74	9.37±7.80	24.34±22.49	<0.001*
Creatinine (mg/dL)	1.10±0.20	1.02±0.18	1.03±0.19	0.846
Hemoglobin (HB)	12.71±1.18	12.36±1.01	12.43±1.10	0.592
	rs10754558			**P value**
	CC (n=50)	**GC (n=40)**	**GG (n=24)**	
Age of onset	40.30±11.0	41.60±11.25	42.08±7.77	0.073
		Sex		
Males	13(26.0%)	12(30.0%)	17(70.8%)	0.907
Females	37(74.0%)	28(70.0%)	7(29.2%)	
ESR (mm/h)	34.68±20.85	28.03±15.60	55.13±28.48	0.159
CRP (mg/l)	8.77±7.58	12.76±7.00	38.40±29.02	<0.001*
Creatinine (mg/dL)	1.01±0.18	1.03±0.18	1.05±0.20	0.328
Hemoglobin (HB)	12.39±1.09	12.39±1.04	12.60±1.10	0.200
	rs6672995			**P value**
	GG (n=82)	**AG (n=18)**	**AA (n=14)**	
Age of onset	41.10±10.81	41.10±1.01	41.43±950	0.994
		Sex		
Males	24(29.3%)	5(27.8%)	3(21.4%)	0.833
Females	58(70.7%)	13(72.2%)	11(78.6%)	
ESR (mm/h)	34.88±20.04	43.39±29.01	38.36±31.70	0.358
CRP (mg/l)	14.23±15.33	21.10±18.01	23.13±31.83	0.129
Creatinine (mg/dL)	1.05±0.19	1.01±0.16	0.93±0.16	0.057
Hemoglobin (HB)	12.46±1.05	12.45±1.12	12.28±1.17	0.848

*P value<0.05. ESR: Erythrocyte sedimentation rate; CRP:C-reactive protein; SD: Standard deviation

demonstrated that there was no substantial relationship between age of onset, sex, ESR, creatinine, and hemoglobin subgroups with rs10754558 genotypes in patients (P>0.05) (Table 5). Finally, the genotype frequency distributions of this polymorphism in controls and the RA groups have coincided with HWE.

Allele Frequency and Genotype Distribution of rs6672995

In the rs6672995 (G>A) variant, the percent of the AA genotype and A allele was slightly greater in the affected group (12.3% for AA genotype and 20.2% for A allele), as compared to the healthy controls (5.8% for AA genotype and 14.6% for A allele); nevertheless, this variance was not important *(*P>0.05). The frequency of GG and AG genotypes in the case group was 71.9% and 15.8%, respectively and these frequencies for the control groups were 76.7% and 17.5%, respectively. Besides, in the RA and the control groups, the frequency of the G allele was 79.8% and 85.4%, respectively. Our results unraveled that the frequency of AA+AG relative to the GG genotype was not much important difference among the patients and the controls (P=0.407) (Table 4). Extra assessment revealed that there was not substantial discrepancy between the RA group with diverse genotypes regardingsome parameters such as the age of onset, gender, ESR, CRP, creatinine, and hemoglobin (P>0.05) (Table 5).

DISCUSSION

Assessment of functional variants in some putative genes associated with multifactorial disease especially polymorphisms in regulatory regions could provide a potent tool to interpret the probable connections between genotype and environmental risk factors in complex disorders including RA. Numerous studies have emphasized the importance of NLRP3 inflammasome in the regulation of the immune system and promotion of inflammation by cleavage and production of some proinflammatory cytokines such as IL-18 and IL-1β (41). Previous publications reported the overexpression of this gene as well as the NLRP3-inflammasome-related proteins in different cells of the immune system of the RA patients and elucidated the contribution of the NLRP3 inflammasome in the RA pathogenesis (27, 30, 42, 43). Furthermore, several specific polymorphisms in this gene have been linked to the RA incidence, disease activity, and response to therapies (31, 44, 45). One research group unveiled that rs4612666 located is in an intronic sequence and rs10754558 situated in the 3UTR site of NLRP3 gene, which could affect the expression level and mRNA consistency, respectively (37). What is more, Shen and colleagues revealed that the G allele in rs10754558 which is situated in miRNA-binding sites involved in the destruction of the miRNA recognition site and modifies the expression of the NLRP3 gene (37). Zhang and coworkers , and through work on gouty arthritis (GA) patients uncovered that subjects with GG and GC genotypes have higher expression of the NLRP3 compared with those with the CC genotype (34). For the first time, in 2009, Villani and coworkers verified that the rare allele (A allele) of rs6672995, located at the regulatory sequence of this gene, enhanced the transcription (38). Varghese and coworkers also emphasized that the A allele of this variant is correlated with increased transcription of the NLRP3 gene in peripheral blood mononuclear cells (PBMCs) (36). To the best of the authors' knowledge, the present research is the first report in Iranians that examines the association among these three NLRP3 variants with the RA susceptibility. Recruiting logistic regression analysis, we discovered that in rs4612666, homozygous CC genotypes compared with the TT genotype increase the risk of the RA (CC vs TT; OR=3.10 [1.78-8.26]). Similarly, combined genotype analyses implied that CC+TC compared with the TT genotype increases the risk of the disease (OR=2.13;

95%CI [1.33-5.50]). Additionally, subjects with the allele C were more frequently afflicted with the RA than persons with the T allele (OR=2.00; 95%CI [1.45-3.10]) (Table 4). In the same vein, the RA individuals with risk allele (C) had an advanced concentration of CRP which was associated with levels of inflammation and the active phase of the disease (Table 5). Previous studies unveiled that the rs10754558 and rs6672995 are associated with the increased expression of the NLRP3 and, therefore, could be a risk factor for the RA; by contrast, in Iranian population, there was not any association between these polymorphisms and susceptibility to the RA (Table 4). In our study, we only established a noteworthy correlation of the G allele in rs10754558 polymorphism with an extraordinary concentration of CRP in the patient group (Table 5). Regarding the relationship of these functional variants with the RA disease, distinctive results have been published from various populations. Two distinct pieces of research in Denmark propounded that rs10754558 and rs4612666 variants are linked with the negative outcome of anti-TNF treatment (45, 46). Another study in the Brazilian population unveiled that C allele and CC genotype in rs10754558 polymorphism was associated with the RA susceptibility in addition to disease severity (32). The next study in the same country demonstrated that rs10754558 (G allele and GG genotype) were higher in SLE patients with the nephritis (25). According to Hanaei et al., GG genotype in rs10754558 variant augmented the risk of ulcerative colitis (UC) in Iranian patients (47). rs4612666 (T allele), in the Chinese population, correlated with increased risk of ankylosing spondylitis (AS) disease and also the efficacy of treatment with etanercept (TNF inhibitors) (33). Villani et al., uncovered that rs6672995 (G allele) is meaningfully greater in cases with the Crohn's disorder (38). Our analysis displayed that rs4612666 is a strong determinant for RA and also there was a substantial correlation between CRP concentration, reminiscing of inflammation and an active disease, and genotypes in rs4612666 and rs10754558. This concerns previous studies pointing out the correlation of CRP with the disease activity (48, 49).

However, replicative study in diverse populations is required before these findings can be approved. Finally, there may be some likely limits in the statistical dependability of our data in the current investigation including a small sample size, thus. As a result, further similar studies with a larger sample size would aid in the validation of the suggested relationships. Additionally, variations not included in our study could have an important role in determining the risk of RA, thus more research is needed.

Conflicts of Interest: None declared.

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