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Association of rs3135500 and rs3135499 Polymorphisms in the MicroRNA-binding Site of Nucleotide-binding Oligomerization Domain 2 (NOD2) Gene with Susceptibility to Rheumatoid Arthritis

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ABSTRACT

The nucleotide-binding oligomerization domain 2 (*NOD2*) is the key regulator of inflammatory responses and has been involved in the pathogenesis of rheumatoid arthritis (RA). Laboratory and in silico evaluations have demonstrated that some polymorphisms in 3′UTR of *NOD2* gene could influence the secondary structure of this region and similarly thermodynamic features of hybridization site and finally deregulate the expression of *NOD2*. In the current study, for the first time, we evaluated the possible association between single nucleotide polymorphisms (SNPs) rs3135500 and rs3135499 in the *NOD2* gene with RA risk in the Iranian population.

One hundred and fifteen patients with RA and 120 healthy subjects were recruited in this case-control study. Genotyping of rs3135500 and rs3135499 polymorphisms were accomplished using the real-time polymerase chain reaction high resolution melting (HRM) method.

We found a substantial association of AA and AG genotypes in rs3135500 with the risk of RA (AA vs GG; OR=5.547; 95%CI [2.564-11.999]; $p<0.001$ and AG vs GG; OR=2.179; 95%CI [1.145-4.147]; $p=0.017$). Moreover, in the patient group, there was a significant relationship between the increased concentration of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) with rs3135500 (A allele) ($p<0.05$). However, there were no important associations

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between rs3135499 with the risk of RA (p>0.05). However, we found a noteworthy association of the C allele in rs3135499 with an increased level of CRP in patients (p>0.05).

Our findings propose a considerable association between NOD2 polymorphisms with increased risk of RA and disease activity.

Keywords: Inflammation; Polymorphism; Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a complex, progressive, heterogeneous, and long-term multi-systemic disorder known as a common type of autoimmune disease.[1,2] RA disorder is characterized by chronic inflammation of the synovium of the joint that leads to painful swelling in and around the joints especially the small joints and consequently leads to bone or cartilage damage. This process eventually results in joint stiffness and deformity.[3,4] The global prevalence of RA is approximately 0.5% to 1% and the incidence increases with the age.[5,6] Considering that RA is a multifactorial disease, several genetic and environmental factors contribute to the pathomechanism of this disease. Recent genome-wide association studies (GWAS) specified several RA-related single nucleotide polymorphisms (SNPs) in multiple genes associated with the immune system such as inflammatory pathways.[7,8] SNPs are the most frequent type of variations in the human genome which exist once almost in every 300 nucleotides and could be associated with multifactorial disorders such as RA.[9,10] MicroRNAs (miRNAs), a class of non-coding RNAs with 18- to 28-nucleotide-long, are involved in various aspects of cellular biology such as inflammatory responses by regulation of gene expression via binding to the 3′-untranslated regions (3′-UTRs) of mRNAs to repress the transcription of target genes.[11,12] Numerous researches have indicated that miRNAs are linked to several aspects of RA pathogenesis.[13,14] Also, several previous studies reported that SNPs located in the 3′UTR of mRNA may modulate miRNA-mRNA interactions by impacting the secondary structure of 3′UTR and thermodynamic features of the hybridization site; this event, finally, dysregulate the expression of the target gene by changing the binding capacity of miRNAs and consequently predispose the individuals to the disease.[15,16] Ample evidence unveiled that amongst the RA-related genes, those that are involved in the inflammatory response, play a crucial role. The nucleotide oligomerization domain 2 (*NOD2*) gene is one of the main regulators of chronic inflammatory conditions.[17,18] NOD2, a cytosolic protein that is expressed in monocytes and macrophages, acts as an activator of nuclear factor-kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), and STAT1 by sensing bacterial lipopolysaccharide (LPS) and ultimately results in the production of pro-inflammatory mediators.[19,20] Several pieces of evidence have unraveled the upregulation of NOD2 in peripheral blood mononuclear cells (PBMCs), synovial fluid T cells (SFTCs), and fibroblast-like synoviocytes (FLS) isolated from RA patients and also showed that these dysregulations were linked with the induction of proinflammatory cytokines and therefore RA pathogenesis.[18,21,22] Furthermore, in vivo analysis revealed that Nod2 deficiency results in reduced joint inflammation and protection against early cartilage damage.[17] Besides, the researchers verified that the down-regulation of expression or loss-of-function mutation in this gene leads to the reduction of proinflammatory cytokines and NF-κB levels.[17,22] A review of the literature and miRSNPs databases such as miRdSNP (http://mirdsnp.ccr.buffalo.edu/), MirSNP (http://bioinfo.bjmu.edu.cn/mirsnp/), and Polymirts (http://compbio.uthsc.edu/miRSNP/) databases showed that rs3135500(G>A) and rs3135499(A>C) polymorphisms, located in the 3′UTR of *NOD2*, are in the miRNA binding site or the vicinity of some miRNAs such as miR-192, miR-158, miR-215, miR-98, and miR-573 for rs3135500 and miR-194, miR-3179, miR-3202, miR-4747, and miR-5196 for rs3135499; these polymorphisms could influence on miRNA-mRNA interaction and finally dysregulate gene expression.[15,23] In the current study, for the first time, we evaluated the probable correlations between these two miRNA binding site polymorphisms in the *NOD2* gene with the RA risk in the Iranian population. Also, we evaluated the interaction among these polymorphisms and some demographic and laboratory characteristics to find their influence on RA susceptibility and disease activity.

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MATERIALS AND METHODS

Study Population

In this case-control study, a total, 115 participants were selected amongst patients referred to the rheumatology division of Alzahra hospitals, Isfahan, Iran according to American College of Rheumatology (ACR) diagnostic criteria. One hundred and twenty healthy controls were also selected from the same population with no signs and symptoms of RA based on negative clinical and laboratory examination. Subjects in the control group have no personal and family history of RA or other immunological and autoimmune diseases. This case-control study was confirmed via the Aja University Research Ethics Committee (UREC) (approval number IR.AJAUMS.REC.1399.099) and written informed consent was filled and approved by all contributors. The participants were interviewed to fill up a structured questionnaire to find the data about the influential factors known to modulate the RA susceptibility risk including sex, age of onset and age at sampling time, height, and weight to calculate body mass index (BMI, calculated as weight (kilogram) divided by height (m) (squared), blood pressure, and family history of RA and other autoimmune disorders. Further to this, we recorded laboratory characteristics such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), white blood cell (WBC) count, hemoglobin, blood urea nitrogen (BUN), platelet count test (PLT), creatinine, fasting blood sugar (FBS), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL). Ultimately, approximately 5 ml of venous blood collected from each subject and drawn into EDTA anticoagulant tubes, and stored at − 20°C for DNA extraction.

SNP Selection and Genotyping

In figure 1, we summarized the in-silico prediction algorithm for SNP selection in the *NOD2* gene. At first, we evaluated the dbSNP database (https://www.ncbi.nlm.nih.gov/snp/) to find SNPs in 3′UTR of *NOD2* and then 1000 Genomes Project was used to screen SNPs according to the criteria of a minor allele frequency (MAF) more than or equal to 0.05 (MAF≥0.05). Finally, to select the SNPs located at miRNAs regulatory elements (MREs). The miRdSNP (http://mirdsnp.ccr.buffalo.edu/) and miRSNP (http://cmbi.bjmu.edu.cn/mirsnp) databases were utilized. Based on these criteria, we reached just 2 polymorphisms (rs3135500 and rs3135499) located in the miRNAs binding site or the vicinity of the binding site of these regulatory molecules.

Genomic DNA was extracted from peripheral blood by PrimePrep Genomic DNA Isolation Kit (GeNetBio, Korea). The yield, purity, and suitability of DNA for polymerase chain reaction high-resolution melting (HRM) method was assessed using spectrophotometry and agarose gel electrophoresis. The forward and reverse primer sequences for amplification of the fragment (134 bp) around rs3135500 polymorphism in the *NOD2* gene were F: AATTGTCAGATGCTGTGCAAATG and R: GCATAAAGTTCACGGCCATGTT and primer sequences for amplification of the fragment (132bp) around rs3135499 polymorphism were F: ACTGAGTGCCTTTTGGTGGA and R: GCCTGGATGGATGAGTCGAG. The HRM method was used to detect different genotypes. This method was carried out using HOTFIREPolEvaGreen HRM Mix (no ROX) HRM PCR kit and analysis accomplished with Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia) under the following conditions: 5 min at 95°C for initial denaturation of the template DNA for the first cycle, 36 cycles of denaturation at 95°C for the 20s, annealing at 59°C (for both polymorphisms) for 30s and extension at 72°C for 20s. Then, HRM analysis was performed by fluorescence acquisition during a temperature ramp from 60°C to 95°C using 0.1°C intervals. To find sample genotypes in HRM analysis as a standard, specific samples (with different melting curves) were subjected to direct Sanger sequencing and their genotypes were distinguished.

Statistical Analyses

The SPSS 25 (Armonk, NY: IBM Corp) was used for statistical analyses. The allele and genotype frequencies of rs3135500 and rs3135499 polymorphisms were tested for Hardy Weinberg equilibrium by the χ^2 test. Logistic regression analysis was performed to examine the correlation between genotypes and RA and compute specific odds ratios (ORs), 95% confidential intervals (CIs), and *P* values. For demographic, clinical, and laboratory characteristics, *p* values were examined using independent sample t-test, Chi-square, or Mann–Whitney test with the significance level of <0.05.

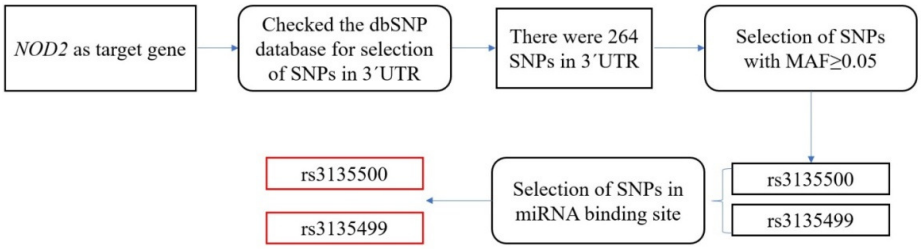

Figure 1. Single nucleotide polymorphism (SNP) selection algorithm

RESULTS

Demographic and Laboratory Characteristics

In this study, we assessed a total of 115 patients (mean age: 47.4±10.446) and 120 control (mean age: 45.39±12.732) to find probable correlations between two miRNA binding site polymorphisms (rs3135500 and rs3135499) in the *NOD2* gene with the RA risk. In table 1, we reported the characteristics of RA subjects and healthy controls. In the RA patient group, the mean age of onset was 41.12±10.39. There was no significant association among case and control subjects about age (p=0.189) and sex (p=0.529) showing that for these characteristics matching was adequate. Between the two groups of contributors, there was a notable difference in terms of BMI and family history of RA and other autoimmune diseases (p<0.001). The results of laboratory tests uncovered that the concentration of ESR, CRP, and creatinine was meaningfully higher in patients compared with healthy controls (p<0.001). In addition, the white blood cell (WBC) count in the patient group was higher than that in the control group (p=0.002). Even so, the concentration of hemoglobin was expressively lower in patients than in healthy participants (p<0.001). The details of the laboratory characteristics of patients with RA and control groups are listed in table 2.

Genotype and Allele Distribution of rs3135500 (G>A)

The frequencies of GG, AG, AA genotypes in the RA patients were 17%, 47%, and 36%, respectively, and the genotype frequencies in the control group were 38%, 48%, and 14%, respectively. Our study elucidated the obvious association between AA, compared with GG, genotype, and RA risk (p<0.001). Likewise, there was a significant difference between the AG genotype compared with the GG genotype in the increased risk of RA (p= 0.017). Our results revealed that combined genotypes including AG+AA (83% in cases compared with 62% in controls) compared to the GG genotype (17% in case group compared with 38% in controls) could increase the risk of RA (p=0.004). Moreover, the frequencies of G and A alleles were 62% and 38% in controls, and 41% and 59% in cases, respectively which demonstrated that the A allele was associated with an increased risk of RA (p=0.013) (Table 3).

Table 1: Baseline characteristics of rheumatoid arthritis (RA) patients and healthy control subjects who participated in this study

Characteristics	Patients	Healthy Controls	*p*
Total number	115	120	--
Age at now	47.4± 10.446	45.39±12.732	0.189
Gender n (%)			
Male	82(71%)	81(68%)	0.529
Female	33(29%)	39(32%)	
Age of onset	41.12±10.39	---	---
BMI	26.20±2.46	24.13±3.30	**<0.001***
SBP	122.43±12.449	120.92±9.744	0.298
DBP	78.48±7.785	78.75±8.282	0.796
Positive family history n (%)	20(17%)	0	**<0.001***

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

Table 2. Laboratory characteristics of patients with rheumatoid arthritis (RA) and healthy control group

	Patients (115)	Healthy Controls (120)	*p*
ESR (mm/h)	37.10±25.544	15.58± 6.918	**<0.001***
CRP (mg/L)	16.30±13.113	4.57±2.817	**<0.001***
White blood cell (10^9/L)	7319.57±2176.254	6578.50±1378.069	**0.002***
Hemoglobin (HB)	12.4304±1.06311	14.3300±1.59055	**<0.001***
PLT (10^9/L)	261.67±71.85103	251.025±66.76680	0.240
Creatinine (mg/dL)	1.0245±.18548	.8593± .17513	**<0.001***
BUN	17.1226±4.67267	16.1167±4.09177	0.080
FBS	96.4435± 15.77317	92.9167±21.95166	0.160
HDL	49.3565± 7.59639	50.4083±11.06550	0.398
LDL	109.97±28.967	107.033±31.28748	0.456
TG	169.10±48.15570	155.57±59.85594	0.058

*$p<0.05$. 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. Association between genotypes and allele frequency of *nucleotide oligomerization domain 2* (*NOD2)* polymorphisms with rheumatoid arthritis (RA) risk

Genotype group	Patients (n=115) n (%)	Controls (n=120) n (%)	OR (95%CI)	*p*
rs3135500				
GG	20(17%)	46(38%)	Reference	
AG	54(47%)	57(48%)	2.179(1.145-4.147)	**0.017***
AA	41(36%)	17(14%)	5.547(2.564-11.999)	**<0.001***
Combined Genotype				
GG	20(17%)	46(38%)	Reference	
AG+AA	95(83%)	74(62%)	2.93 (1.55-5.72)	**0.004***
Allele				
G	94(41%)	149(62%)	Reference	
A	136(59%)	91(38%)	2.364 (1.6-3.4)	**0.013***
rs3135499				
AA	48(42%)	51(43%)	Reference	
AC	40(35%)	47(39%)	0.904(0.487-1.676)	0.769
CC	27(23%)	22(18%)	1.30 (0.62-2.75)	0.487
Combined Genotype				
AA	48(42%)	51(43%)	Reference	
AC+CC	67(58%)	69(57%)	1.03 (0.59-1.79)	0.99
Allele				
A	136(59%)	149(62%)	Reference	
C	94(41%)	91(38%)	1.13 (0.76-1.66)	0.571

*$p < 0.05$

Finally, we reported that the genotype distribution of rs3135500 (G>A) polymorphism in two groups was in agreement with Hardy–Weinberg equilibrium. Besides, stratification based on some laboratory characteristics revealed that patients with different genotypes have a significantly different mean concentration of CRP and ESR. In detail, patients with risk allele (A) had a higher concentration of CRP and ESR (p<0.001). However, there was no significant association between the stratification of the age of onset, gender, hemoglobin, and creatinine with different genotypes of this polymorphism (p>0.05) (Table 4).

Table 4. Association of *nucleotide oligomerization domain 2* (*NOD2)* polymorphisms with various parameters of rheumatoid arthritis (RA)

	rs3135500			*p*
	GG (n=20)	**AG (n=54)**	**AA (n=41)**	
Age of onset	37.76± 11.206	41.40± 12.617	43.07± 10.242	0.320
Sex				
Males	17(85%)	35 (65%)	30(73%)	0.221
Females	3 (15%)	19 (35%)	11(27%)	
ESR (mm/h)	22.10± 6.640	29.76± 13.736	54.10±26.385	**<0.001***
CRP (mg/L)	5.70±5.021	11.28±4.576	28.07±14.767	**<0.001***
Creatinine (mg/dL)	0.9835±0.20964	1.0341±0.17567	1.0320±0.18779	0.556
Hemoglobin (HB)	12.1050±.96599	12.3870±1.11525	12.6463±1.01319	0.161
	rs3135499			
	AA (n =48)	**AC (n =40)**	**CC (n =27)**	***p***
Age of onset	44.37±11.384	35.85±10.410	42.76±11.193	**0.012***
Sex				
Males	35(73%)	26(65%)	21(77%)	0.499
Females	13(27%)	14(35%)	6(23%)	
ESR (mm/h)	34.15±19.955	36.55±22.555	43.19±26.278	0.247
CRP (mg/l)	11.86±7.703	16.42±11.776	24.00±18.456	**<0.001***
Creatinine (mg/dL)	1.0087±.20914	1.0275±.16224	1.0481±.17677	0.676
Hemoglobin (HB)	12.2917±1.11696	12.5275±1.05587	12.5333±0.98332	0.500

* p< 0.05. ESR: Erythrocyte sedimentation rate; CRP:C‑reactive protein; SD: Standard deviation

Genotype and Allele Distribution of rs3135499 (A>C)

The genotype distribution of rs3135499 polymorphism in case and control groups was in agreement with Hardy–Weinberg equilibrium. Among the RA cases, the frequency of the AA, AC, and CC was 42%, 35%, and 23%, respectively. In the control group, the frequency of the rs3135499 genotypes was 43% for AA, 39% for AC, and 18% for CC. In addition, the frequencies of A and C alleles were 59% and 41% in cases, and 62% and 38% in the control group, respectively. Comparison of genotype and allele frequencies of the rs3135499 polymorphism between the case and control groups disclosed no significant differences (p>0.05). Also, the comparison of combined genotypes unveiled that the CC + AC genotypes compared to the AA genotype were not significantly different between case and control groups

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(p>0.05). However, the mean concentration of CRP in the patient group was significantly different in genotype stratification (p<0.001). In details, the concentration of CRP in patients with AA, AC, and CC genotype were 11.86±7.703, 16.42±11.776, and 24.00±18.456, respectively. This means that patients with C allele have a higher concentration of CRP. Similarly, the age of onset in the patient group was meaningfully different in genotype stratification (p=0.012). Nevertheless, there was no significant association between the stratification of the sex, hemoglobin, ESR, and creatinine concentrations with this polymorphism (p>0.05) (Table 4).

DISCUSSION

Numerous studies have emphasized the fact that a large number of single nucleotide polymorphisms (SNPs) loci affect the susceptibility to autoimmune disorders.[24,25] Between these polymorphisms, functional polymorphisms such as SNPs located in the 3´UTR of immune and inflammatory-related genes could contribute to the disruption of miRNA recognition elements (MREs) or create new sites leading to up or down-regulation of these genes and consequently may be remarkably associated with RA risk. NOD2 is the key regulator of immune and inflammatory responses; not surprisingly, dysregulation of this gene takes part in chronic inflammatory conditions such as Crohn's disease (CD) and ulcerative colitis (UC).[26,27]

Mutations in *NOD2* or altered expression of this gene was observed in several diseases such as Blau syndrome (BS), inflammatory bowel disease (IBD), early-onset sarcoidosis (EOS), Ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), and RA (18, 26, 28-31).[18,26,28-31] Convincing lines of evidence unraveled that polymorphisms in this gene were correlated with different diseases. For example, some non-synonymous polymorphisms including Pro268Ser, Arg702Trp, Gly908Arg as well as frame shift mutations like L1007fsincC and 3020insC have investigated in different disorders such as CD and colorectal and gastric cancers.[32-36]

Based on literature review and different bioinformatics algorithms and databases (e.g., targetscan, miRbase, miRanda, and miRTarBase), this gene is targeted by several miRNAs such as miR-158, miR-215, miR-573, miR-573, miR-122, miR-192, miR-495, miR-512, miR-671, and miR-98.[23,37] The existence of some polymorphisms in the 3´UTR of the *NOD2* gene could influence the secondary structure of this region and similarly thermodynamic features of the hybridization site and finally deregulate the expression of *NOD2*.

For the first time in the Iranian population, we set out to evaluate the correlation of 2 functional polymorphisms (rs3135500 and rs3135499) which are located in the miRNA binding site of this *NOD2* gene with the RA risk. In our study, logistic regression analysis revealed that in rs3135500, homozygous AA genotypes and heterozygous AG genotypes, compared with the GG genotype, increase the risk of RA (AA vs GG; OR=5.547; 95%CI [2.564-11.999] and AG vs GG; OR=2.179; 95%CI [1.145-4.147]). Likewise, combinational genotype analyses indicated that AA+AG compared with the GG genotype increases the risk of RA disease (OR= 2.93; 95%CI [1.55-5.72]). Initially, Landi and colleagues reported that rs3135500 is correlated with sporadic colorectal cancer (CRC). Additionally, in the Iranian population, two different studies worked on the association between this variant and CRC risk and reported different results. Ahangari et al reported that AA genotype was correlated with increased risk of CRC and Chaleshi et al observed a lack of association between this polymorphism and risk of CRC in the Iranian population.[38,39] A study in the population of Caucasian adults population discovered that the A allele at rs3135500 was meaningfully associated with an increased risk of asthma.[40] Cao and coworkers assessed the PBMCs from multiple system atrophy (MSA) patients and uncovered that patients with the "A" allele of rs3135500 had higher mRNA *NOD2* levels which might increase the risk of MSA.[41] Similarly, a study in the Turkish population indicated that this polymorphism could be related to chronic obstructive pulmonary disease (COPD) progression.[42] However, in the present study, there was no association between genotypes and allele frequency of rs3135499 with RA risk (p>0.05). Nevertheless, Enevold and colleagues reported that this polymorphism was associated with the increase in the time to relapse of multiple sclerosis.[43] In the other study carried out in the Chinese population, Cai et al demonstrated that the C allele of rs3135499 was correlated with an increased risk of asthma.[44]

Furthermore, in our study, we found a remarkable association of the A allele in rs3135500 polymorphism with a high concentration of ESR and CRP in the patient group (p<0.05). What's more, the C allele in rs3135499 was associated with an increased level of CRP in patients (p<0.05). The higher level of these factors indicates the level of inflammation in the body and refers to the correlation of these polymorphisms with higher disease activity.[45,46]

In summary, this study provided evidence that the *NOD2* gene rs3135500 polymorphisms were associated with RA risk and it seemed that A allele in rs3135500 and C allele in rs3135499 polymorphism was connected with disease activity. However, because of the limited sample size and different genetic background, replicative studies in the Iranian population and different populations are necessary to validate these results.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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