




## Single nucleotide polymorphism rs5029937 in TNFAIP3 gene is correlated with risk of rheumatoid arthritis

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### Abstract

**Background:** Rheumatoid arthritis (RA) is a progressive and common autoimmune disease with multifactorial etiology. Several pieces of research show that genetic factors play a major role in the incidence of RA. Several genome-wide association studies (GWAS) have identified the tumor necrosis factor alpha inducible protein 3 (TNFAIP3) genes as one of the candidate loci. The TNFAIP3 gene encoding ubiquitin-editing protein A20 which restricts B cell survival and prevents autoimmunity. Previous studies have indicated that single nucleotide polymorphisms (SNPs) in the TNFAIP3 gene are correlated with several autoimmune disorders. In the present study, we assessed the possible association between SNP rs5029937 (intronic variant) in the TNFAIP3 gene with RA risk in the Iranian population.

**Methods:** A case-control study using 50 RA patients and 50 control subjects was undertaken to evaluate rs5029937 (G>T) genotypes using real-time PCR high resolution melting method (HRM). The SPSS22 was used for statistical analyses and the significance level was set at  $P < 0.05$ .

**Results:** Logistic regression analysis demonstrates that homozygous TT + heterozygous TG genotypes compared with GG genotype increase the risk of RA (TT+TG vs GG;  $P = 0.004$ , OR= 3.46; 95%CI [1.492-8.075]). Also, individuals with allele T were more frequently affected with RA than subjects with G allele (T vs G;  $P = 0.004$ , OR= 2.61; 95%CI [1.382-4.919]).

**Conclusion:** Our findings propose a substantial correlation between rs5029937 (G>T) polymorphism and RA risk in Iranian population.

**Keywords:** Rheumatoid Arthritis, TNFAIP3 Gene, Single-nucleotide Polymorphism, Autoimmune Disorder, HRM

**Conflicts of Interest:** None declared

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### Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation and joint damage and eventually swelling and motor disability in the patient's

joints (1). This disease is the most common form of chronic inflammatory arthritis and often leads to joint damage and physical disability. Since RA is a systemic disease, it

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#### ↑What is “already known” in this topic:

Studies have shown that *TNFAIP3* is fundamental for regulating inflammation by dismissing TNF-induced *NFKB* responses through tumor necrosis factor receptor (*TNFR*), *TLR*, *IL1R* and *NOD2*. Variations in the *TNFAIP3* gene, as a key regulator of inflammatory signaling pathways, seem to dramatically contribute to autoimmunity.

#### →What this article adds:

Our results demonstrate that combination of TT+TG compared with the GG genotype in rs5029937 polymorphism increased the risk of disease. On the other hand, individuals with allele T were more frequently affected with RA than subjects with G allele.

can cause many extra-articular manifestations, some of which include fatigue, subcutaneous nodules, lung involvement, pericarditis, peripheral neuropathy, vasculitis, and hematologic disorders (2). The worldwide prevalence of RA is approximately between 0.5 – 1 % and its prevalence increases markedly with age (3, 4). RA has multifactorial etiology, mirroring as interaction of several inherited and environmental factors are associated with an increased risk for this disease (5). Several lines of evidence show that genetic determinants are critically involved in the incidence of RA. Twin studies showed that 50–60% of RA onset could be attributed to genetic factors (6, 7). Recently with advances in genotyping and sequencing methods, studies reached several loci associated with RA risk. For example, genome-wide association studies (GWAS) reported more than 100 risk loci for RA in European and Asian descents (8, 9). Most of these loci are involved in immunological function; interestingly, there is considerable overlap in these loci between RA and other autoimmune disorders (10). In this way, recent genome-wide association studies (GWAS) determined multiple RA-related single nucleotide polymorphisms (SNPs). The most frequent type of variations in the human genome is single nucleotide polymorphism (SNP) that they exist once almost in every 300 nucleotides (11-13). The tumor necrosis factor alpha inducible protein 3 gene (*TNFAIP3*) is one of the identified candidate loci by several GWAS that conducted to recognize new predisposing genes of the main autoimmune diseases (14, 15). *TNFAIP3* encodes the cytoplasmatic zinc finger A20 protein that inhibits *NFKB* activation and TNF-mediated apoptosis (16). Studies in knockout mice show that *TNFAIP3* is important for limiting inflammation by terminating TNF-induced *NFKB* responses through tumor necrosis factor receptor (*TNFR*), *TLR*, *IL1R* and *NOD2* (17). Decreased A20 expression predisposes to autoimmunity as is shown in knockout mice, which exhibits elevated numbers of germinal center B cells, autoantibodies and glomerular immunoglobulin deposits (18, 19). Variations in the *TNFAIP3* gene, as a key regulator of inflammatory signaling pathways, seem to dramatically contribute to autoimmunity (20). In this way, several association studies demonstrated that polymorphisms in this gene are associated with autoimmune disorders including RA (21-23), systemic lupus erythematosus (SLE) (24, 25), type 1 diabetes mellitus (26), systemic sclerosis (27, 28), Graves' disease (GD) (29), Behcet's disease (BD) (30), and primary Sjogren's syndrome (pSS) (31). One of these functional polymorphisms is rs5029937 (G>T) that located in intron 2 of *TNFAIP3* gene. A meta-analysis study from 10 case-control research revealed that rs5029937 polymorphism increased the risk of RA among Caucasians (21). Furthermore previous studies demonstrated that this variant is associated with SLE in Europeans and Asian populations (24, 32). In the present study, we assessed the possible association between SNP rs5029937 (G>T) in the *TNFAIP3* gene with RA risk in the Iranian population for the first time.

## Methods

### Study population and sample preparation

In this case-control study, a total of 50 unrelated subjects with RA as a case (mean age: 51.46±10.10) and unrelated 50 healthy subjects as a control group (mean age: 49.52±13.85) were included in Isfahan city of Iran. Patients were recruited from the Alzahra hospitals. All the RA subjects met the diagnostic criteria published by the American College of Rheumatology (ACR). All persons in control group had no symptoms or any history of RA or other autoimmune diseases. The study participants were interviewed and data on sex, age (at sampling time) and age of onset, body mass index (BMI, calculated as weight [kg] divided by height [m] squared), blood pressure, smoking status, the presence of diabetes mellitus (DM), thyroid disease and family history of RA and other autoimmune disorders were achieved using a organized questionnaire. Also, we recorded laboratory characteristics such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cell (WBC), hemoglobin, platelet count test (PLT), creatinine, blood urea nitrogen (BUN), fasting blood sugar (FBS), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), rheumatoid factor (RF). This study was permitted by the university ethics board and all contributors gave written informed consent.

### DNA extraction and genotyping of polymorphism

Approximately, 5 ml of peripheral blood was prepared into ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes from each subjects and stored at – 20°C for DNA isolation. Genomic DNA was extracted from 200 µL of venous blood samples using GenetBio kit (Korea) consistent with the instruction manual. The purity and concentration of all genomic DNA samples were assessed by agarose gel electrophoresis and spectroscopy at wavelengths of 260 and 280 nm respectively, and then DNA was stored at –20 °C until genotyping by real-time polymerase chain reaction high-resolution melting (HRM) method.

The HRM method was performed to evaluate rs5029937 polymorphism genotypes. HRM was performed using HOT FIREPol EvaGreen HRM Mix (no ROX) HRM PCR kit which contains HOT FIREPol® DNA Polymerase, 5x EvaGreen® HRM buffer, 12.5 mM MgCl<sub>2</sub>, dNTPs, Bovine serum albumin (BSA), and EvaGreen dye (Solis BioDyne Estonia). Analysis carried out with Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). The forward and reverse primer sequences for the 236-bp fragment that spanned the rs5029937 in *TNFAIP3* gene were TTTTGCACCTTGCCAAAGGAGA and AAAAAGCACCTGGGTGTCTAAA, respectively. The thermal profile of the reaction is as follows: 5 min denaturation at 95 °C, 40 cycles of 95° C for 10 s, 60°C °C for 30 s and 72°C for 20 s. The melting curve is generated by increasing between 60 °C and 95 °C at 0.1°C/s. The melting curve was produced by the reduction in fluorescence with the increase in the temperature; and in analysis, nucleotide changes result in different curve patterns. For using sample genotypes in HRM analysis as a stand-

ard, specific samples (with different curves) were subjected to direct Sanger sequencing and their correct genotypes were determined.

### Statistical analyses

The SPSS 22 (IBM, Armonk, NY: IBM Corp) was used for statistical analyses. The allele and genotype frequencies were tested for Hardy Weinberg equilibrium by the  $\chi^2$  test. Logistic regression analysis was accomplished to investigate the association between genotypes and RA and calculate specific odds ratios (ORs), 95% confidential intervals (CIs), and P values. Other analyses carried out using independent sample t-test, ANOVA test, Chi-square ( $\chi^2$ ) or Mann-Whitney test. The significance level was set at  $P < 0.05$ .

## Results

### Demographic and clinical characteristics

In order to assess the association among rs5029937 polymorphism with RA risk, we analyzed 100 total subjects in case and control groups; 50 subjects (30 female and 20 male with a mean age of onset:  $42.68 \pm 14.01$ ) in the case and 58 (27 female and 23 male) healthy subjects in the healthy group. The distributions of selected characteristics of the cases and controls are presented in Table 1. There was no considerable association between case and control group regarding age ( $p=0.334$ ) and gender ( $p=0.342$ ), representative that matching for these factors was sufficient. However, cases and controls did not contrast on BMI ( $p=0.394$ ) and systolic blood pressure (SBP) ( $p=0.537$ )

and diastolic blood pressure (DBP) ( $p=0.918$ ). Ten (20%) patients had DM while healthy controls did not have DM ( $p=0.001$ ) and ten (20%) patients had a positive family history where controls did not have a history of any autoimmune disease ( $p=0.001$ ). Based on laboratory tests, ESR and CRP were meaningfully higher in cases than in healthy controls ( $p < 0.001$ ). Positive RF was detected in 50 (100%) patients. Other laboratory factors including WBC, FBS, hemoglobin, creatinine, BUN, PLT, HDL, LDL, and TG were not significantly different between patients and healthy controls ( $p > 0.05$ ). The detailed laboratory characteristics of patients with RA and healthy controls are listed in Table 2.

### Genotype and allele distribution

The analysis demonstrates that the genotype distribution of rs5029937 polymorphism in two groups was in agreement with Hardy-Weinberg equilibrium. The frequency of GG genotype in case and control group was 32% and 62% respectively and frequency of TG genotype in case and control group was 60% and 38% respectively and finally frequency of TT genotype in case group was 8% but was not found in control group (0%). When we compared the combined genotype, our results demonstrated that the TT + TG compared to the GG genotype increase the risk of RA ( $p=0.004$ ) (Table 3). Also, in allele distribution, we found that T allele has a high frequency in the case group (38%) compared to the control group (19%) and this allele (T) is associated with RA risk ( $p=0.004$ ). In addition, our analysis revealed that the median concentration of ESR

Table 1. Baseline characteristics of RA patients and control subjects participated in the study

Characteristics	Patients	Controls	p
Total number	50	50	
Age	$51.46 \pm 10.10$	$49.52 \pm 13.85$	0.334
Gender n (%)			
Male	20 (40%)	23 (46%)	0.342
Female	30 (60%)	27 (54%)	
Age of onset	$42.68 \pm 14.01$	--	--
BMI	$25.87 \pm 3.85$	$24.89 \pm 6.98$	0.394
SBP	$119.20 \pm 14.19$	$120.4 \pm 10.49$	0.537
DBP	$80.01 \pm 8.08$	$79.80 \pm 9.68$	0.918
Positive family history n (%)	10 (20%)	0	0.001
Diabetes mellitus	10 (20%)	0	0.001
Thyroid disease	5 (10%)	0	0.021
Smoker	5 (10%)	0	0.021

Data are mean  $\pm$  SD, or n (%). \*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 controls group

	Patients (50)	Controls (50)	p
ESR (mm/h)	$37.27 \pm 32.21$	$15.63 \pm 6.93$	$< 0.001^*$
CRP (mg/l)	$19.70 \pm 20.20$	$4.02 \pm 2.43$	$< 0.001^*$
White blood cell ( $10^9/l$ )	$6.83 \pm 2.41$	$6.84 \pm 1.62$	0.981
Hemoglobin	$13.67 \pm 1.62$	$14.27 \pm 1.55$	0.072
PLT ( $10^9/l$ )	$228.69 \pm 70.64$	$221.14 \pm 62.82$	0.212
Creatinine (mg/dL)	$0.94 \pm 0.17$	$0.87 \pm 0.21$	0.081
BUN	$16.52 \pm 4.93$	$15.83 \pm 4.54$	0.473
FBS	$96.30 \pm 20.18$	$98.18 \pm 42.54$	0.782
HDL	$50.66 \pm 9.80$	$47.5 \pm 12.01$	0.151
LDL	$106.39 \pm 31.57$	$106.16 \pm 38.76$	0.976
TG	$141.65 \pm 54.78$	$147.93 \pm 69.81$	0.611
Positive RF	50 (100%)	-	-

Data are mean  $\pm$  SD, or n (%). \*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.** Association between genotypes and allele frequency with RA risk

Genotype group	Patients (n = 50) N (%)	Controls (n = 50) N (%)	p	OR (95%CI)
GG	16 (32%)	31 (62%)	Reference	
TG+TT	34 (68%)	19 (38 %)	0.004	3.46 (1.492-8.075)
Allele				
G	62(62%)	81(81%)	Reference	
T	38(38%)	19(19%)	0.004	2.613 (1.382-4.919)

\* P value &lt; 0.05

**Table 4.** Stratification analyzes of the TNFAIP3 polymorphism (rs5029937) in patients.

Genotype group	TG+TT (n=34)	GG (n=16)	p
Age of onset	42.91±14.09	42.2±13.89	0.621
Sex			
Males	12	8	0.061
Females	22	8	
ESR (mm/h)	39.29±28.16	32.9±22.36	0.033*
CRP (mg/l)	21.68±21.58	16.89±16.93	0.022*
Creatinine (mg/dL)	0.96±0.216	0.85±0.154	0.079
BMI	25.37±2.85	26.17±3.23	0.237

Data are mean±SD, or n (%). \* P value &lt; 0.05. ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; BMI: Body mass index; SD: Standard deviation

and CRP in the patient group is significantly different in genotype stratification ( $p < 0.05$ ). However, there was no significant correlation between other clinical factors including sex, age, BMI, creatinine and, age of onset with this polymorphism ( $p > 0.05$ ) (Table 4).

### Discussion

To the best of our knowledge, this study is the first research in the Iranian population that considers the association amongst *TNFAIP3* polymorphism, rs5029937, with the RA risk. The *TNFAIP3* encoding ubiquitin-editing protein A20 with restricts B cell survival and prevents autoimmunity (33). Based on several previous studies, *TNFAIP3* gene and their polymorphisms is one of the crucial genetic factors in inflammation and autoimmune disease, especially in RA (26, 27, 34, 35).

In our work, logistic regression analysis demonstrates that combination of TT+TG compared with the GG genotype increases the risk of disease (OR= 3.46; 95%CI [1.492-8.075]). On the other hand, individuals with allele T were more frequently affected with RA than subjects with G allele (OR= 2.61; 95%CI [1.382-4.919]) (Table 3). Our finding was consistent with studies on Caucasian populations. In a meta-analysis performed by Zhang and colleagues in 2017, they assessed correlation among *TNFAIP3* gene polymorphism and RA, stratification analysis of ethnicity revealed that in rs5029937 polymorphism, GT+TT vs. GG genotypes increased the risk of RA in total ( $p < 0.001$ , OR= 1.42) and specifically in Caucasians ( $p = 0.004$ , OR= 1.43) while there was not association in Asian ethnicity (just 1 study in Korea) ( $p = 0.185$ ). Furthermore in this meta-analysis study, revealed same results for allele frequency and they demonstrated that T vs. G allele increased risk of RA in Caucasian ( $p = 0.002$ , OR=1.43) but not in Asian ethnicity (just 1 study in Korea) ( $p = 0.168$ ) (36). The other met-analysis study on this polymorphism carried out by Shen et al and their results was consistent with Zhang study (22). On the other hand some studies demonstrated association between rs5029937 and SLE in different type of populations. As instance,

Kim et al revealed that TT+TG genotypes and T allele increased risk of SLE in Korean population (25) and Han et al demonstrate same results in Han Chinese population (37). Furthermore in the patient group, we found a significant correlation between ESR and CRP concentration and rs2075876 polymorphism ( $p < 0.05$ ) (Table 4). The level of these factors indicates levels of inflammation in the body and refers to active disease. This result demonstrates association of risk allele with severity of disease.

The causes for the some inconsistent results may be due to variances in ethnic background. Nevertheless, performing replicative researches in other population is a necessity to validate these results. We believe that our work would further justify the role of the *TNFAIP3* gene and rs5029937 variant in RA susceptibility. Finally, in this work, probably, some probable restrictions in the statistical validity of our results including small population size exist, so additional association studies in larger sample size would help to confirm the proposed associations. Also, other polymorphisms that were not included in our study might be involved in determining the risk of RA, thus future studies are necessary.

### Conclusion

In conclusion, our findings propose a substantial correlation between rs5029937 (G>T) polymorphism and RA risk in Iranian population.

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### Ethics

The study was approved by Isfahan University of Medical Sciences Ethics board (approval number IR.MUI.MED.REC.1397.010) and written informed consent was filled and signed by all participants.

### Conflict of Interests

The authors declare that they have no competing interests.

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