Single Nucleotide Polymorphism rs2075876 in AIRE Gene Is a Strong Rheumatoid Arthritis Determinant

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Abstract- 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 autoimmune regulator (*AIRE*) gene as one of the candidate loci. This gene encodes a transcription factor, which is involved in the presentation of self-antigens and the negative selection of self-reactive T-cells in the thymus. Studies have indicated that single nucleotide polymorphisms (SNPs) in the *AIRE* gene can change the gene expression and/or function. In the present study, we assessed the possible association between SNP rs2075876 (intronic variant) in the *AIRE* gene with RA risk in the Iranian population. A case-control study using 56 RA patients and 58 control subjects was undertaken to evaluate rs2075876 genotypes using the real-time PCR high resolution melting method (HRM). Logistic regression analysis demonstrates that homozygous AA and heterozygous AG genotypes compared with GG genotype increase the risk of RA (AA vs. GG; OR=16.43; 95% CI [5.33-50.71] and AG vs. GG; OR=3.21; 95% CI [1.22-8.45]). Also, individuals with allele A were more frequently affected with RA than subjects with G allele (OR=5.81; 95% CI [3.28-10.30]). Furthermore, in the patient group, we found a significant correlation between erythrocyte sedimentation rate and C-reactive protein concentration with rs2075876 polymorphism (P<0.05). Our findings propose a substantial correlation between rs2075876 polymorphism and RA risk.

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Keywords: Rheumatoid arthritis; Autoimmune regulator (*AIRE)* gene; Single-nucleotide polymorphism; Autoimmune disorder

Introduction

Rheumatoid arthritis (RA) is a long-term, progressive, and common autoimmune disease. It causes inflammation, painful swelling in and around the joints and other body organs (1,2). The worldwide prevalence of RA is approximately between 0.5-1%, and its prevalence increases markedly with age (3,4). RA, like most autoimmune diseases, displays a striking imbalance between the sexes (female/male ratio is around 3:1) (5,6). RA has multifactorial etiology, mirroring as the interaction of several inherited and environmental factors are associated with an increased risk for this disease (7). 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 (8,9). On the other hand, some works demonstrated that having a positive family history of RA increases the risk of developing RA by about 3-5 times (10). 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 (11,12). Most of these loci are involved in immunological function; interestingly, there is considerable overlap in these loci between RA and other autoimmune disorders (13). The autoimmune regulator (*AIRE*) gene is one of the identified candidate loci by several GWAS that conducted to recognize new predisposing genes of the

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main autoimmune diseases.

Variations in the *AIRE* gene, as an immune regulator, seem to contribute to autoimmunity (14) dramatically. The protein product of this gene is a transcription factor that is expressed in the medulla (inner part) of the thymus. AIRE protein is involved in the negative selection of self-reactive T-cells in the thymus as well as the induction of regulatory T-cells by interacting with CREB binding protein; evidently, this protein has a pivotal role in the maintenance of central tolerance (15,16). The cytogenetic location of this gene is in the 21q22.3 region and encodes a protein with 545 amino acids (17). More than 90 mutations in the *AIRE* gene have been recognized in subjects with autoimmune polyendocrine syndrome type 1 (APS-1), also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), which is a complex rare autosomal recessive disorder (16,18). Recent studies have indicated that single nucleotide polymorphisms (SNPs) in the *AIRE* gene can change gene expression and/or function (19). In this way, several association studies demonstrated that polymorphisms in this gene are associated with autoimmune disorders including RA (19,20), alopecia areata and Universalis (21,22), systemic sclerosis (23), myasthenia gravis (24), type 1 diabetes mellitus (25), systemic lupus erythematosus (SLE) (26), vitiligo (27), autoimmune hepatitis (28) and another disease such as melanoma (29). One of these functional polymorphisms is rs2075876 (c.653-387G> A) that located in intron 5 of AIRE gene (20). The bioinformatics analysis demonstrated the decreased expression of the *AIRE* gene by the mutated allele (risk allele; A) of rs2075876 compared with wild type allele (G) (30). Previous studies indicate a significant correlation between this variant and RA and alopecia areata in different populations such as the Chinese population (21,31). In the present study, we assessed the possible association between SNP rs2075876 in the *AIRE* gene with RA risk in the Iranian population for the first time. Also, some demographic and laboratory characteristics in relation to rs2075876 genotypes and their influence on RA susceptibility was worked out.

Materials and Methods

Study population and sample preparation

Based on previous studies on this polymorphism and sample size formula (N: $\frac{(z1-z2)^2\,[p1(1-p1)+p2(1-p2)]}{(p1-p2)^2}$) and other statistical calculations in this case-control study consisted of 56 unrelated subjects with RA (mean age: 52.46±11.10) and unrelated 58 healthy subjects as a control group (mean age: 49.52±14.95) in Isfahan city of Iran. Patients were recruited from the Alzahra hospitals, the biggest affiliated Hospital of Isfahan University of Medical Sciences. All the RA patients met the diagnostic criteria created by the American College of Rheumatology (ACR; revised in 2010) (32). Healthy controls, the volunteers who had negative RA laboratory test results, with no symptoms or personal and family history of RA or other immunological and autoimmune disorders were recruited from the Alzahra hospitals. 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, the presence of diabetes mellitus (DM) and family history of RA and other autoimmune conditions were obtained using a structured 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 approved by the university ethics board, and all participants gave written informed consent.

DNA extraction and genotyping of polymorphism

Approximately 5 ml of venous blood was collected into ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes from each participant and stored at 20° C for DNA isolation. Genomic DNA was extracted from 200 µL of peripheral 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.

The real-time polymerase chain reaction high-resolution melting (HRM) method was used to determine rs2075876 polymorphism genotypes. HRM was performed using a Type-it HRM PCR kit, contains HotStarTaq plus DNA polymerase and EvaGreen dye (Qiagen Germany), and analysis carried out with Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). The forward and reverse primer sequences for the 201-bp fragment that spanned the rs2075876 in the AIRE gene were TGGGAGGCTTGAAATGACAGAA and GACACGTGGGAGACAGCTG, 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 for 30 s and 72° C for 20 s. The melting curve is generated by increasing between 65° C and 95° C at 0.1° C/s. Finally, melting curves were normalized among the two temperatures to determine the specimens with known genotypes as standard. For utilizing sample genotypes in HRM analysis as a standard, some samples 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 χ^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 (χ^2) or Mann–Whitney test. The significance level was set at $P<0.05$.

Results

Demographic and clinical characteristics

To assess the association between rs2075876 polymorphism with RA incidence, we analyzed 114 total subjects in case and control groups; 56 patients (34 female and 22 male with a mean age of onset: 40.55±12.91) in the case and 58 (41 female and 17 male) healthy subjects in the control group. Table 1 shows the demographic and clinical characteristics of patients included in the study. There was no substantial correlation between case and control group regarding age ($P=0.234$) and gender ($P=0.262$), demonstrating that for these variables matching was adequate. As shown, patients have higher BMI compared with control groups ($P=0.025$). Five (8.9%) patients had DM whereas controls did not have DM ($P=0.02$) and eight (14.3%) patients had a positive family history, where controls did not have a history of any autoimmune disease ($P=0.013$). However, patients and controls did not differ on systolic blood pressure (SBP) and diastolic blood pressure (DBP) ($P>0.05$). Based on laboratory tests, ESR, CRP, and HDL were significantly higher in patients than in healthy controls ($P<0.05$). Positive RF was observed in 56 (100%) patients. Other laboratory factors, including WBC, FBS, hemoglobin, creatinine, BUN, PLT, LDL, and TG, were not significantly different between patients and healthy controls ($P>0.05$). The laboratory characteristics of patients with RA and healthy controls are presented in Table 2.

Genotype and allele distribution

The analysis demonstrates that the genotype distribution of rs2075876 polymorphism in two groups was in agreement with the Hardy-Weinberg equilibrium. The frequencies of the GG, AG, and AA genotypes were 17.9%, 30.3%, and 51.8% in the patient group, and 58.6%, 31%, and 10.3% in the control group, respectively. Our analyses demonstrated the significant association of AA and AG genotype with the risk of RA in our subjects (AA vs. GG; $P<0.001$, and AG vs. GG; $P=0.018$). When we compared the combined genotype, our results demonstrated that the AA+AG compared to the GG genotype increases the risk of RA ($P<0.001$). Also, in allele distribution, we found that the A allele has a high frequency in the case group (66.9%) compared to the control group (25.8%), and the A allele is associated with RA risk ($P<0.001$). The distribution of genotype and allele frequency are shown in Table 3. In addition, our analysis revealed that the mean concentration of ESR 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).

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

Characteristics		Patients	Controls	*P*
Total number		56	58	
Age		52.46±11.10	49.52±14.95	0.234
Gender n (%)	**Male**	22 (39.3%)	17(29.3%)	0.262
	Female	34 (60.7%)	41(70.7%)	
Age of onset		40.55±12.91	--	--
BMI		26.07±2.85	23.87±6.67	0.025*
SBP		119.55±13.46	120.95±10.02	0.531
DBP		80.27±7.89	79.83±9.08	0.783
Positive family history n (%)		8 (14.3%)	0	0.013*
Diabetes mellitus		5(8.9%)	0	0.02*

Data are mean±SD, or n (%). *$P<0.05$. RA: Rheumatoid arthritis; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

Table 2. Laboratory characteristics of patients with RA and controls group.

	Patients (56)	Controls (58)	*P*
ESR (mm/h)	37.2±31.65	14.33±7.36	<0.001*
CRP (mg/l)	19.72±24.20	4.06±2.53	<0.001*
White blood cell (10^9/1)	6.74±2.41	6.73±1.62	0.975
Hemoglobin	13.49±1.71	14.07±1.60	0.069
PLT(10^9/1)	238.79±70.64	222.04±67.92	0.208
Creatinine (mg/dL)	0.94±0187	0.87±0.21	0.074
BUN	16.51±5.28	14.89±4.96	0.097
FBS	92.87±17.18	88.29±21.22	0.209
HDL	51.79±8.97	46.84±11.45	0.012*
LDL	111.91±30.09	104.50±36.60	0.244
TG	166.87±49.43	150.28±65.34	0.131
Positive RF	56(100%)	0	<0.001*

Data are mean±SD, or n (%).*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 with RA risk.

Genotype group		Patients (n=56) n (%)	Controls (n=58) n (%)	OR (95%CI)	*P*
GG		10(17.9%)	34(58.6%)	Reference	--
AG		17(30.3%)	18(31%)	3.21(1.22-8.45)	0.018*
AA		29(51.8%)	6(10.3%)	16.43(5.33-50.71)	<0.001*
Combined Genotype	**GG**	10(17.9%)	34(58.6%)	Reference	--
	AA+AG	46(82.1%)	24(41.3%)	6.52 (2.32-13.18)	<0.001*
Allele	**G**	37(33.01%)	86(74.1%)	Reference	--
	A	75(66.9%)	30(25.8%)	5.81(3.28-10.30)	<0.001*

* P<0.05

Table 4. Stratification analyzes the AIRE polymorphism (rs2075876) in patients.

Genotype group		AA (n=29)	AG (n=17)	GG (n=10)	*P*
Age of onset		40.21±12.19	44.7±12.55	44.7±12.55	0.511
Sex	**Males**	12(41.4)	12(70.6)	5(50)	0.541
	Females	17(58.6)	5(29.4)	5(50)	
ESR (mm/h)		34.69±28.16	45.76±36.16	29.9±33.36	0.037*
CRP (mg/l)		17.28±16.88	28.47±35.48	11.89±14.93	0.026*
Creatinine (mg/dL)		0.97±0.216	0.95±0.137	0.85±0.154	0.079
BMI		26.37±2.85	26.10±2.92	25.17±2.83	0.337

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

Discussion

To the best of our knowledge, this study is the first research in the Iranian population that investigates the association between *AIRE* polymorphism, rs2075876, with the RA risk. Association studies reported a correlation between polymorphism in this gene and different autoimmune diseases. One of these polymorphisms is rs2075876, which is an intronic variant. Bioinformatics assessments display that several nucleotide sequences around this polymorphism show a high degree of conservation amongst mammalian species. Furthermore, in silico analysis has revealed that the mutated allele (risk allele; A) of rs2075876 reduced the expression level of *AIRE* compared with wild type allele (G allele) (30). This might dysregulate the expression of auto-antigens, resulting in the failure of negative selection and, finally, survival of autoreactive T cells.

In our work, logistic regression analysis demonstrates that homozygous AA and heterozygous AG genotype compared with the GG genotype increase the risk of RA (AA vs. GG; OR=16.43; 95% CI [5.33-50.71] and AG vs. GG; OR=3.21; 95%CI [1.22-8.45]). Also, combined genotype analyses indicate that AA+AG compared with the GG genotype increases the risk of disease (OR=6.52; 95% CI [2.32-13.18]). On the other hand, individuals

with allele A were more frequently affected with RA than subjects with G allele (OR=5.81; 95% CI [3.28-10.30]) (Table 3). Therefore regards to the importance of *the AIRE* gene in autoimmune disease and the effect of these polymorphisms on gene function and our data, it can be concluded that the risk allele in rs2075876 could increase the risk of RA disease in the Iranian population.

Our finding was consistent with a study in the Chinese population carried out by Feng *et al.*, in 2014 (31). Another study in the Chinese population by Shao *et al*., demonstrates the same results (33). Although Li X *et al.*, demonstrated reverse results, which means the G allele increases the risk of RA in the Chinese population (34). On the other hand, studies on the Spanish Caucasian and the Japanese population in two different surveys showed that there was not a significant correlation between this polymorphism (rs2075876) with RA risk (35). Toraih EA *et al*., demonstrated that GG genotype is more frequent in patients with alopecia areata (GG versus AA: OR=16.1) (21). The reasons for the contradictory results may be due to differences in ethnic background and different types of autoimmune diseases. However, performing replicative studies in every population is a necessity to validate these results. 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. In this context, Feng *et al*., reported that this variant just correlated with CRP concentration (31).

We believe that our work would further justify the role of the *AIRE* gene and rs2075876 variant in RA susceptibility. Finally, in this work, probably, some possible limitations in the statistical validity of our results such as small population size exist, so further association studies in larger sample size would help to confirm the suggested correlations. Also, other polymorphisms that were not included in our study might be involved in determining the risk of RA, thus future studies are necessary.

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