PADI4 polymorphisms in Iranian patients with rheumatoid arthritis

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ABSTRACT

Aim: Rheumatoid arthritis (RA) is a chronic autoimmune disease, which affects many tissues and organs, but majorly attacks synovial joints. Beyond the major histocompatibility complex (MHC) genes, peptidyl arginine deiminase type IV (PADI4) has been suggested to be associated with RA susceptibility. Evidence regarding the association of PADI4 single nucleotide polymorphisms (SNPs) and RA is controversial, thus we conducted this large-scale case-control study to assess the association of rs874881 and rs11203367 PADI4 SNPs with susceptibility to RA.

Materials and Methods: Study population (including 665 RA patients and 392 sex-, age-, and ethnicity--matched healthy controls) were enrolled from Rheumatology Research Center of Tehran University of Medical Sciences, Shariati hospital.

Results: Allele or genotype frequencies of the investigated PADI4 SNPs were not different between RA patients and healthy subjects; genotypes (expressed as odds ratios) of rs11203367 [TT 0.98 (0.68-1.4), CT 0.93 (0.71-1.24), P value > 0.05] and rs874881 [CG 0.97 (0.73-1.29), GG 0.98 (0.68-1.41), P value > 0.05] did not affect RA risk. Disease severity score DAS28, RF and anti-CCP antibodies of RA patients were not different between various genotypes of PADI4 SNPs.

Conclusions: These findings were similar for haplotypes and diplotypes of rs11203367 and rs874881 PADI4 SNPs. In conclusion, in this case-control study with sufficient sample size to detect associations, we observed that PADI4 SNPs rs11203367 and rs874881 do not significantly determine RA susceptibility; which is in line with studies of some European populations. It seems RA pathogenesis might be different among various ethnicities, which encourage us to consider these differences in developing therapeutic interventions for the management of patients.

Keywords: Rheumatoid arthritis; PADI4 gene polymorphisms

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INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation and joint destruction. Although this disease affects up to 1 % of the worldwide population, the etiology remains mainly unknown. Both genetic and environmental factors seem to play important roles in the pathogenesis of RA[1]. Genetic factors are responsible for up to 60% of RA susceptibility. Human leukocyte antigen DRB1 (HLA-DRB1) is known as the most powerful genetic factor associated with RA accounting for one-third of RA genetic susceptibility[2]. Recently a non--HLA gene called peptidyl arginine deiminase type IV (PADI4) has been shown to be associated with RA in many studies. PADI4 is a member of the family of related genes encoding enzymes, which catalyze the conversion of arginine residues to citrulline during the posttranslational modification of peptides[3-5]. Citrullinated proteins (CCP) were identified in the synovial fluid of RA patients; antibodies against these peptides (anti-CCP) are highly specific for RA diagnosis. Anti--CCP antibodies have been shown to be predictive for the development of RA and are also associated with the severity of the disease[6].

Therefore PADI4 gene has become an interesting non HLA-gene candidate to be associated with RA. For the first time Suzuki et al discovered the association between PADI4 gene polymorphisms and RA susceptibility in a Japanese population in 2003[7]; since then various studies in different ethnic groups have been performed to confirm this finding. The studies conducted in East Asian, North American and German populations reported the PADI4 gene as susceptibility loci for RA; which is consistent with the Japanese study. Nevertheless, findings of some European cohorts have been inconsistent[8-10]. Studies in Spanish, Swedish and UK populations failed to find the association between PADI4 and RA[11-13]. Two meta-analyses published in 2006 and 2007 concluded that PADI4 polymorphisms are associated with RA susceptibility in European populations with a lesser degree than Asians[14,15]. Another meta-analysis in 2013 investigated the studies conducted in China and Egypt suggested that PADI4 polymorphisms represent an important risk factor for RA in Asians[16].

Due to the observed controversy between ethnic groups, this study was designed to investigate the association between RA and the PADI4 gene SNPs in a large Iranian population.

PATIENTS AND METHODS

STUDY POPULATION

A total of 1057 unrelated Iranian individuals, including 665 RA patients and 392 healthy controls matched by sex, age and ethnicity were enrolled from March 2012 to April 2014. All the RA patients met the American College of Rheumatology 1987 criteria[17]. All the patients were recruited from Rheumatology Research Center (RRC) of Tehran University of Medical Sciences (TUMS), Shariati hospital. The patients comprised 569 women and 96 men, with mean (±SD) age of 50.77 (±11.9) years. The healthy controls were recruited from Iranian Blood Transfusion Organization and included of 324 women and 68 men, with mean (±SD) age of 49.98 (±13.25) years. Control subjects and their family didn't have any autoimmune or rheumatic disease. This study was approved by the ethics committee of Tehran University of Medical Sciences, and all individuals in both groups filled informed consent forms.

GENOTYPING OF PADI4 SNPS

Genomic DNA was extracted from peripheral blood samples with the standard phenol-chloroform-proteinase-K method[18]. Samples were genotyped for two SNPs (PADI4-90, rs11203367) and (PADI4-92, rs874881) in PADI4 gene. Genotyping was performed using ABI StepOnePlus Real-Time PCR system by allelic discrimination TaqMan genotyping assay (Applied, Biosystems Foster city, USA). The allelic call was performed by the analysis of allelic discrimination plots, using ABI SDS V 2.2 software. The genotype distributions of cases and controls were found to be in Hardy--Weinberg equilibrium.

MEASUREMENT OF ANTI-CCP AND RF

The serum concentration of both anti-CCP and RF were measured for RA patients and healthy controls using the ELISA test (ORGENTEC Diagnostika, GMBH, and Germany).

DISEASE ACTIVITY SCORE-28 [DAS28] SCORING

The RA activity was measured by using the DAS28 index[19,20]. DAS28 is a weighted multi-dimensional indicator that uses a physician's assessment of the joints, the patient's overall self-assessment of disease activity, and a laboratory marker of inflammation (CRP or ESR). This score (ranging from 0-9.4) includes the total number of tender joints (up to 28), total number of swollen joints (up to 28), and general health assessment on visual analogue scale (VAS). RA disease activity is interpreted as low (DAS28 < 3.2), moderate (3.2 < DAS28 < 5.1) or as high disease activity (DAS28 > 5.1).

STATISTICAL ANALYSIS

Descriptive analysis was performed so that continuous variables were tested for normality by means of Shapiro-Wilk test. Data with normal distribution are expressed as mean ± SD while non-normally distributed ones are reported as median (interquartile range [IQR]). Differences between groups regarding normally distributed continuous variables were tested by t-test, and one-way ANOVA followed by post hoc analysis where indicated. For variables without normal distribution, Kruskal-Wallis and Mann-whitney U tests were employed. For measuring the association of PADI4 SNPs and RA; Chi-square test was performed as cross-tabulation, and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. Fisher s Exact and Monte-Carlo tests were used where cell frequencies of cross-tabulations were insufficient. For haplotype analysis of PADI4 SNPs we used SHEsis online

software[21]. To obtain diplotypes we employed Microsoft™ Office Excel 2007 using CONCATENATE formula to generate the genotype codes according to our method described in our previous study[22]. Then, we coded each diplotype by conditional (IF) formula, and exported them to SPSS for further risk estimation analyses. P-value less than 0.05 was considered significant. Data were analyzed by SPSS software version 16 (SPSS Inc, Chicago, Illinois, USA). To achieve the observed power of study firstly for single SNP differences we used the sample size formula of case-control studies where N is sample size of patients; α is the observed P-value; p_0 stands for minor allele frequency (MAF) of SNP in controls and p_1 is MAF in patients; C represents controls/patients ratio; and β will be calculated as the observed type II error; finally the observed power is $1-\beta$. Also for Chi-square tests (genotype differences), we used the formula $power=1-\chi'^2$, where χ'^2 is a left-tail non-central Chi-square distribution with degree of freedom (df); performed by SPSS version 16 *compute variable* formula (1-NCDF.CHISQ (quant, df, nc). The required sample size for achieving a significant difference for each gene with minimum power of 80% was calculated.

RESULTS

BASELINE CHARACTERISTICS

Study population was a total of 1057 unrelated Iranian individuals, including 665 RA patients and 392 healthy controls matched by sex, age and ethnicity (Table I). No significant difference was found between RA patients and healthy subjects regarding age (P=0.834), sex (P=0.207), and ethnicities (P=0.086). The median (IQR) of RF was 66.8 (203) and 11 (10) in RA patients and healthy subjects, respectively; which showed a significant difference (P<0.0001). Also the median (IQR) anti-CCP of RA patients (66.3 [265]) was significantly (P<0.0001) higher than healthy subjects (11.5 [7]).

TABLE I. BASELINE CHARACTERISTICS OF STUDIED POPULATION

	RA patients	Healthy controls
Number	665	392
Age (years)	50.77 ± 11.9	49.98 ± 13.2
Female/Male	569/96	324/68
Anti-CCP (U/ml)	403.6 ± 1047.5	13.92 ± 16.49
RF (U/ml)	302.7 ± 697.2	13.7 ± 12.2

GENOTYPE AND ALLELE FREQUENCY OF PADI4 POLYMORPHISMS

The allele frequencies and genotype distribution of PADI4 are summarized in Table II. No significant differences in allele or genotype frequencies between RA patients and controls were seen for rs11203367. Genotypes of rs11203367 [TT 0.98 (0.68-1.4), CT 0.93 (0.71-1.24), P value > 0.05] were not significantly associated with RA. Moreover, no significant differences in allele or genotype frequencies between RA patients and controls were seen for rs874881. Similarly, genotypes of rs874881 [CG 0.97 (0.73-1.29), GG 0.98 (0.68-1.41), P value > 0.05] were not statistically linked with RA susceptibility.

HAPLOTYPE AND DIPLOTYPE FREQUENCY OF PADI4 POLYMORPHISMS

The frequencies of rs11203367 and rs874881 PADI4 SNPs haplotype and diplotype are displayed in Tables III and IV, respectively. CC and TG haplotypes were not significantly associated with RA susceptibility (Table III). Also CC/CC, CT/CG, and TT/GG did not significantly influence RA risk (Table IV).

ASSOCIATION BETWEEN ANTI-CCP, RF, DAS28 AND PADI4 POLYMORPHISMS

RF, anti-CCP and DAS28 are known to be associated with RA severity. Therefore, the median RA, anti-CCP and DAS28 levels were tested for difference between genotypes of PADI4 SNPs. RF, anti-CCP and DAS28 showed no significant differences between the aforesaid genotypes of rs11203367 and rs874881 (data are not shown).

DISCUSSION

In this study, we evaluated the PADI4 polymorphisms and RA susceptibility in a large Iranian population. PADI4 is a non-HLA gene suggested to be associated with RA. Our results showed no significant association regarding PADI4 SNPs rs11203367 and rs874881 with RA susceptibility.

Our findings are in line with the studies performed on French, Spanish, British and German populations

TABLE II. ASSOCIATIONS BETWEEN ALLELES AND GENOTYPES OF PADI4 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS) IN RHEUMATOID ARTHRITIS (RA)

SNP	Allele/Genotype	RA patients Number (%)	Controls Number (%)	OR (95% CI)	P-value	Power	SS§
rs11203367	T	568 (42.5)	337 (43)	1.01 (0.85-1.21)	0.855	*52.62%	268820†
	C (reference)	766 (57.5)	447 (57)	1	–		
	Hardy-Weinberg	χ^2=0.42 P=0.52	χ^2=0.008 P=0.93				
	TT	125 (18.7)	72 (18.4)	0.98 (0.68-1.41)	0.932		
	CT	318 (47.7)	193 (49.2)	0.93 (0.71-1.24)	0.636	#67.62%	–
	CC (reference)	224 (33.6)	127 (32.4)	1	–		
rs874881	C (reference)	763 (57.2)	446 (56.9)	1	0.889	*51%	222023†
	G	571 (42.8)	338 (43.1)	1.01 (0.85-1.2)	–		
	Hardy-Weinberg	χ^2=0.08 P=0.78	χ^2=0.0008 P=0.98				
	CC (reference)	220 (33)	127 (32.4)	1	0.839		
	CG	323 (48.4)	192 (49)	0.97 (0.73-1.29)	0.915	#61.92%	–
	GG	124 (18.6)	73 (18.6)	0.98 (0.68-1.41)	–		

*we used the sample size formula of case-control studies $N=\left[Z_{1-(\alpha/2)}\times\sqrt{(1-(1/C))\times(\bar{p}\bar{q})}+Z_{1-\beta}\times\sqrt{(p_1q_1)+(p_0q_0/C)}\right]^2/(p_1-p_2)^2$ where N is sample size of patients; α is the observed P-value; p_0 stands for minor allele frequency (MAF) of SNP in controls and p_1 is MAF of aforesaid SNP in patients; C represents controls/patients ratio; $\bar{p}=(p_1+(C\times p_0))/(1+C)$ and β will be calculated as the observed type II error; finally the observed power is *1-β*.

for χ^2 tests (genotype differences), we used the formula *power* $=1-\chi'^2_{df,\lambda}$ where $\chi'^2_{df,\lambda}$ is a left-tail non-central Chi-square distribution with degree of freedom (*df*) and non-centrality parameter $\lambda=Nw^2=N\times(\sum_{i=1}^{m}((p_{0i}-p_{1i})^2/p_{0i}))$; *compute variable formula* (1-NCDF.CHISQ (quant, df, nc).

§Required sample size (SS) was calculated by the sample size formula of case-control studies (*). †Very small effect size (OR≈1) attributes to the lack of difference and calculated required sample size is not practical, thus it seems that 667 RA patients and 392 healthy controls is a powerful size for drawing a lack of association conclusion

showing no association between PADI4 SNPs and RA[11,12,23,24]. However, our results do not confirm findings of East Asian studies regarding association between PADI4 SNPs and RA susceptibility[7-9,25,26]. Suzuki et al evaluated several SNPs distributed along the PADI4 gene locus in Japanese population and found PADI4-94 (rs2240340) as the most important SNP linked with RA susceptibility[7]. Several studies were conducted among East Asian and Caucasian populations to confirm this finding; however, their results were controversial.

Considering these discrepancies, Lee et al conducted a meta-analysis on three Asian and six European studies to assess whether the PADI4 polymorphisms influence the susceptibility to RA[15]. It showed a significant association of PADI4-94 (rs2240340), PADI4-104 (rs1748033), and PADI4-90 (rs11203367) with RA in Asian population but in Europeans only PADI4-94 (rs2240340) was found to be significantly associated with RA. This meta-analysis concluded that the PADI4 polymorphisms might be a significant risk factor for RA in both Asian and European populations; however, PADI4 SNPs seem to be more important contributors for RA susceptibility in Asians. Consequently, it was proposed that the European studies had small sample sizes and were not powerful enough to detect the genetic association, therefor Burr et al performed the PADI4-94 and RA association study in a large UK population (a sample of over 19000 UK subjects); interestingly no evidence of association between PADI4-94 SNP and RA was found[27]. Recently, Hou et al designed a meta-analysis on 21 studies including Asian studies and previous European studies. They studied six SNPs

TABLE III. ASSOCIATION BETWEEN HAPLOTYPES OF RS11203367 AND RS874881 PADI4 SINGLE NUCLEOTIDE POLYMORPHISMS AND RHEUMATOID ARTHRITIS (RA)

Haplotypes*	RA patients Number (%)	Controls Number (%)	P-value	OR (CI95%)
CC	762 (57.1)	444(56.6)	0.879	1.014 (0.848-1.212)
TG	567(42.5)	335 (42.7)	0.879	0.986 (0.825-1.179)

*Haplotype frequencies<0.03 in both controls and cases have been excluded

TABLE IV. ASSOCIATION BETWEEN DIPLOTYPES OF RS11203367 AND RS11203367 PADI4 SINGLE NUCLEOTIDE POLYMORPHISMS AND RHEUMATOID ARTHRITIS (RA)

Diplotypes	RA patients Number (%)	Controls Number (%)	P-value	OR (CI95%)
CC/CC	220 (33.0)	125 (31.9)	0.713	1.051 (0.805-1.373)
CT/CG	318 (47.7)	190 (48.5)	0.803	0.969 (0.755-1.243)
TT/GG	124 (18.6)	72 (18.4)	0.928	1.015 (0.736-1.400)

(PADI4-94, 104, 92, 90, 89 and 100); all six SNPs were significantly associated with RA in Asian populations. Three SNPs (PADI4, 90, 89) showed significant association in Europeans, while the remaining (PADI4-94, 92, 100) were not associated with RA in European subjects. Finally, they suggested PADI4 SNPs represent a significant risk factor for RA, especially in Asians[16].

Our data showed lack of association between these gene markers and RA susceptibility. In "lack of association" studies one may inquire into whether these non-significant findings are mainly due to lack of enough power (small sample size, small effect size, high variation) of the study. Thus we calculated the observed power of our study design and chi-square test so that we found a low power ranging 50-70% for our findings. Furthermore we investigated the source of this low power; interestingly our calculations were indicating a very small effect size (odds ratios ranging 0.98--1.01). Also the sample size calculations showed that in order to find a significant difference for these small effect sizes we need more than 200.000 RA patients and 200.000 healthy controls, which is not feasible and scientifically reasonable. Actually, where effect sizes of genetic association studies are very small, we cannot calculate a valid estimate of study power since in these cases type I and type II errors are nearly identical. In these situations, narrow confidence intervals are better descriptors for study power. Hoenig and Heisey elucidate the abuse of observed power calculations in biomedical studies and recommend some practical methods to examine the study power in their seminal paper[28].

In this study we faced some limitations as we could not estimate other SNPs of PADI4 gene. Also, individuals of this case-control were enrolled from a referral hospital, thus we suggest conducting more population--based studies to overcome possible selection bias. However, we tried to overcome population stratification by investigating the ethnicities of participants, and we tried to remove the genetic artifacts through PCR testing by exploring both RA cases and healthy controls in each PCR run. Moreover, the reasonable sample size of this study and studying the haplotype-diplotype variants are the other strength of our study.

In summary, PADI4-90 (rs11203367) and PADI4--92 (rs874881) SNPs do not affect the susceptibility to RA among Iranian population. By considering these data together, we hypothesize ethnicities influence PADI4 polymorphisms and RA pathogenesis, which intrigue us to conduct studies in various ethnics for exploring the possible differences of RA susceptibility between populations; so that it might pave the way to design more individualized therapeutic interventions for management of RA patients.

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