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Original article

Evaluation of the association of single nucleotide polymorphisms in DDP4 and CDK5RAP2 genes with rheumatoid arthritis susceptibility in Iranian population

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ABSTRACT

Background: Rheumatoid arthritis (RA) is known as a chronic autoimmune inflammatory disorder, which is characterized mainly by the progressive inflammation and destruction of the joints. In the pathogenesis of RA, a variety of cell types such as lymphocyte, dendritic cells, osteoclasts and synovial fibroblasts are involved. Genetic proneness has been implicated in the pathogenesis of RA. The aim of this study was to evaluate the association of single nucleotide polymorphisms (SNPs) in *DPP4* and *CDK5RAP2* genes and risk of RA in Iranian population.
Methods: For genotyping, 623 RA patients and 412 healthy subjects were recruited. Genetic analysis of *DPP4* gene rs12617656 and *CDK5RAP2* gene rs12379034 polymorphisms was conducted using TaqMan allelic discrimination (for rs12617656) and ARMS-PCR (for rs12379034) methods.
Results: Experiments demonstrated that alleles and genotypes of both SNPs were represented equally in RA patients and controls. Statistical analysis revealed that none of the rs12617656 and rs12379034 SNPs had significant differences in prevalence of both alleles and genotypes between RA patients and healthy controls.
Conclusions: It appears that gene polymorphisms of *DPP4* and *CDK5RAP2* are not involved in the pathogenesis of RA in Iranian population.

1. Introduction

RA is a chronic multifactorial disorder with involvement of the small diarthrodial joints of the hands and feet. Both genetics and environmental factors are the important contributing factors in the pathogenesis of RA [1–4]. Recently, determining roles of genetics in RA susceptibility have been explored mostly by genome-wide association studies (GWAS). Human leukocyte antigen (HLA) account for one-third of the genetic contributing factors for RA pathogenesis in addition to non-HLA components, which are increasingly being detected by genome-wide scannings [5].

DPP4 gene encodes dipeptidyl peptidase-4 (DPP4) or cluster of differentiation 26 (CD26), which is a membranous glycoprotein and is expressed on the surface of immune and non-immune cells with enzymatic activity of cleaving proline containing dipeptides. Its role has been clarified in some previous studies as an immune regulator and its serum levels and cell surface form have been sur-

Abbreviations: RA, rheumatoid arthritis; SNP, single nucleotide polymorphism; GWAS, genome-wide association study; HLA, human leukocyte antigen; DPP4, dipeptidyl peptidase-4; CD26, cluster of differentiation 26; CDK-5, Cyclin-dependent kinase 5; CDK5RAP2, Cyclin-dependent kinase 5 regulatory subunit–associated protein 2; ACR, American college of rheumatology; EDTA, ethylenediaminetetraacetic acid; ARMS-PCR, amplification refractory mutation system-polymerase chain reaction; OR, odds ration; HWE, Hardy–Weinberg equilibrium; ESR, erythrocyte sedimentation rate; DAS28, disease activity score in 28 joints; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; RANK, receptor activator of nuclear factor κB.

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veyed in several autoimmune diseases including RA [6,7]. The strong association of *DPP4* gene rs12617656 SNP with RA risk was demonstrated in a GWAS, indicating how genes can affect RA proneness in Han Chinese populations [8]. We also hypothesized that this polymorphism might take part in RA susceptibility and possibly in the progression of the disease in Iranian population.

Cyclin-dependent kinase 5 (CDK-5) regulatory subunit–associated protein 2 (CDK5RAP2) has an important role in differentiation process of neuronal system by forming the microtubule nucleator via gamma-tubulin ring complex recruitment to centrosomes [9]. This is a vital stage in brain evolution and mutations in the attributed genes during brain development result in mitosis division disturbance in neurons, culminating in microcephaly [10]. Surprisingly, a GWAS detected a strong association of rs12379034 SNP in *CDK5RAP2* gene with the above-mentioned function with RA susceptibility, introducing the association of a neuronal-related gene with an autoimmune disease for the first time [8].

Given the important roles of the *DPP4* gene in RA predisposition and how *CDK5RAP2* acts in immune and neuronal systems of RA patients, we were interested in surveying the possible connections between the two mentioned polymorphisms in these genes and RA risk in Iranian population.

2. Subjects and methods

2.1. Patients and controls

Our study population was composed of 623 (524 females and 99 male) RA patients recruited from the Rheumatology Clinic of Shariati Hospital and 412 (356 females and 56 male) healthy unrelated subjects without any history of autoimmune diseases. The RA patients who met the 1997 revised American College of Rheumatology (ACR) classification criteria [11] were delicately matched with control group considering age, sex, and ethnicity. Alongside genotyping of SNPs, phenotypical characteristics of RA patients were obtained to investigate the relation between two polymorphisms and clinical features. Written inform consent was taken by all the subjects and the protocol of this study was approved by the Ethics Committee of Tehran University of Medical Sciences. The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

2.2. Genotyping

Blood samples were taken from patients and healthy individuals into ethylenediaminetetraacetic acid (EDTA) containing tubes and genomic DNA was extracted from peripheral leukocytes applying the phenol-chloroform method [12]. The extracted DNA was stored at −20 °C until the proper time for the experiments. Genotyping was performed using StepOnePlus Real-Time PCR System and TaqMan MGB-based allelic discrimination method (both Applied Biosystems, Foster City, CA, USA) for rs12617656 and Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) for rs12379034. The ARMS-PCR technique [13] is a precise and rapid method, detecting a specific SNP in a large number of SNPs and is based on a mutation or mismatched nucleotide(s) at 3′ end of the primer. Three primers including two forward (mutant and wild-type) and one reverse common primer (Table 1) were designed by Primer 3 online tool (http://primer3.ut.ee). The ARMS-PCR was conducted in a 15 μL reaction containing 2 μL DNA template, 5U Taq DNA polymerase (SinaClon, Iran), 2.5 μL 10× PCR buffer (SinaClon, Iran), 0.25 μL 10 mM dNTP (SinaClon, Iran) and 1 μL of each specific primer (10 pmol/μL). Two internal control primers with 0.12 μL volume in each reaction was also utilized. The following primers were used as internal control, forward: 5′ TGCTCAGCCAGTTTGACTTA 3′ and reverse: 5′ CCTGCAGGTATATTTTGGCG 3′. PCR was performed as follow: an initial denaturation at 94 °C for 3 min, 40 cycles for DNA amplification consist of 30 s at 94 °C, 30 s at 63.6 °C, 30 s 72 °C and a final extension at 72 °C for 10 min. PCR products were electrophoresed in 2% agarose gel stained with DNA safe staining (SinaClon, Iran) and were observed through UV transilluminator (Fig. 1). We chose a few samples for sequencing to confirm the accuracy of the amplification (Fig. 2).

Table 1
Primers used in ARMS-PCR for *CDK5RAP2* gene rs12379034 genotyping, amplicon size and melting temperature of each reaction.

Primer	Sequence	Amplicon size (bp)	Temp (°C)
NF	AGCTAGTCCATGAGGACAAATga	220	57.4
MF	AGCTAGTCCATGAGGACAAATgg	220	57.4
RC	CTTACTTGATCCGTCCCACA	220	58.5
Seq	AGGAATCAGAAGGTCCAGAA	282	58.7

NF, Normal Forward primer; MF, Mutant Forward primer; RC, Reverse Common primer; Seq, sequence primer.

To perform TaqMan MGB-based allelic discrimination, all PCR reactions mixture contained approximately 25–75 ng of DNA, 5 μL Taq-Man Master Mix containing Taq DNA polymerase and dNTPs (Applied Biosystems, Foster City, USA), 0.25 μL Taq-Man Genotyping Assay mix containing primers and FAM or VIC labeled probes (Applied Biosystems, Foster City, USA), and distilled water for a final volume of 10 μL. Thermocyclic conditions of PCR were: initially 60 °C for 30 s and then 95 °C for 10 min, and subsequently 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min), and finally 60 °C for 30 s.

2.3. Statistical analysis

All statistical analyses were performed using SPSS, version 21 (IBM Inc, Chicago, IL, USA). The generalized linear model (i.e. logistic regression) and chi-square tests were used to evaluate the association of alleles and genotypes of SNPs with disease status and clinical manifestations, respectively. Odds ratios (ORs) as the effect size with 95% confidence intervals (95% CI) were calculated in each group. The control group was evaluated for Hardy–Weinberg Equilibrium (HWE) in each locus using SHEsis online tool [14]. P values less than 0.05 were considered as statistically significant.

3. Results

Demographic and laboratory information of RA patients and healthy controls are shown in Table 2.

The genotype distribution of *DPP4* gene rs12617656 and *CDK5RAP2* gene rs12379034 SNPs in the healthy controls demonstrated no significant deviation from the HWE ($P = 0.124$ and 0.256, respectively). As shown in Table 3, the global major allele of T was regarded as the reference allele for rs12617656 SNP according to NCBI database. The C allele of rs12617656 SNP was distributed almost equally between patients and controls (33.7% *vs*. 32.4%). Therefore, this allele had no significantly different distribution between RA patients and healthy controls (OR = 0.95, CI: 0.74–1.23; $P = 0.74$). On the other side, considering the TT genotype as the reference, genotypes of rs12617656 SNP did not represent significantly different prevalence between cases and controls. The CT genotype of this SNP was less represented in RA patients in comparison to healthy subjects (43.5% *vs*. 46.1%); hence the difference was not statistically significant (OR = 0.90,

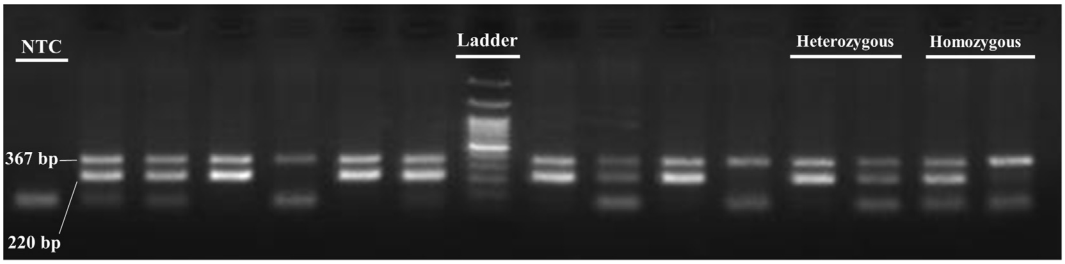

Fig. 1. Genotyping models of rs12379034 SNP in *CKD5RAP2* gene obtained from ARMS-PCR implementations. First line depicts the internal control with 367 bp product size. The second line illustrated DNA bands of with 220 bp of size. For homozygous samples, there is one 220 bp band, while two 220 bp bands are seen for heterozygous samples. From right to left, the first one is for mutant allele and the second is for wild-type DNA. DNA ladder and No template control (NTC) are also depicted.

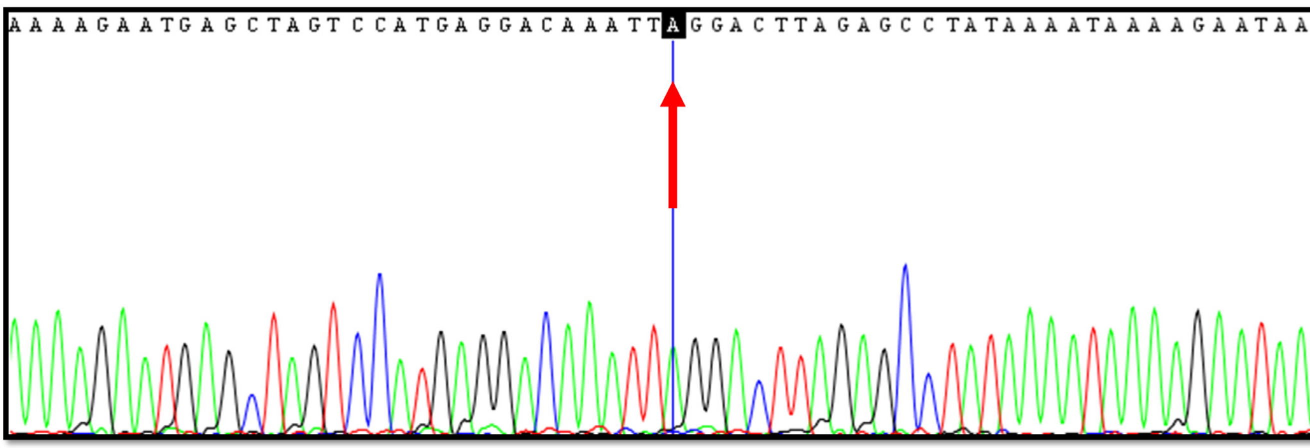

Fig. 2. Sequencing analysis of ARMS-PCR products of *CDK5RAP2* gene rs12379034 SNP in a patient, demonstrating the A allele. The results of sequencing were in accordance with agarose gel electrophoresis of ARMS-PCR products.

Table 2
Demographic data and laboratory specifications of RA patients and healthy controls.

Characteristic	RA patients (n = 623) N (%)	Healthy individuals (n = 412) N (%)
Male	99 (16%)	56 (13.5%)
Female	524 (84%)	356 (86.5%)
Age	50.32 ± 12.05*	42.69 ± 12.64
RF	82.23 ± 97.14	–
Anti-CCP	129.91 ± 219.83	–
DAS28	2.92 ± 1.2	–
ESR	17.98 ± 15.53	–

RA, Rheumatoid Arthritis; RF, Rheumatoid Factor; anti-CCP, anti-Cyclic Citrullinated Peptide Antibody; DAS28, Disease Activity Score for 28 Joints; ESR, Erythrocyte Sedimentation Rate.

* Data are represented as Mean ± Standard Deviation.

CI: 0.63–1.29; P = 0.59). As such, the CC genotype was not differently distributed between cases and controls. As the dominant genotype, the CC + CT pattern did not show significantly different prevalence between RA cases and healthy controls.

On the other hand, the A allele of rs12379034 SNP was supposed as the reference allele (Table 3). The G allele of this SNP demonstrated the same frequency in cases and controls with insignificant difference (OR = 1.05, CI: 0.83–1.31; P = 0.67). The AA genotype of rs12379034 SNP was considered as the reference genotype. The AG genotype of this SNP was more frequent in RA patients than in controls (30.4% *vs.* 27.7%); however, the difference was not significant (OR = 1.24, CI: 0.95–1.61; P = 0.11). Despite the GG genotype was more frequent in healthy subjects, the prevalence difference was not statistically significant. The AG + GG model was the dominant genotype and it was expressed more

Table 3
Allele and genotype frequencies of *DPP4* gene rs12617656 and *CDK5RAP2* gene rs12379034 SNPs in RA patients and healthy controls.

dbSNP	Alleles/genotypes	Case (n = 623) N (%)	Control (n = 412) N (%)	*P*	OR (95% CI)
DPP4	T	851 (66.3)	557 (67.6)	Reference	–
rs12617656	C	395 (33.7)	267 (32.4)	0.74	0.95 (.74–1.23)
	TT	290 (46.6)	182 (44.2)	Reference	–
	CT	271 (43.5)	190 (46.1)	0.59	0.90 (0.63–1.29)
	CC	62 (9.9)	40 (9.7)	0.94	0.97 (0.54–1.77)
	CC + CT	333 (53.5)	230 (55.9)	0.63	0.92 (0.65–1.29)
HWE			P = 0.124		
CDK5RAP2	A	1013 (81.3)	676 (82)	Reference	–
rs12379034	G	233 (18.7)	148 (18)	0.67	1.05 (0.83–1.31)
	AA	412 (66.2)	281 (68.2)	Reference	–
	AG	189 (30.4)	114 (27.7)	0.11	1.24 (0.95–1.61)
	GG	22 (3.4)	17 (4.1)	0.23	0.66 (0.33–1.31)
	AG + GG	211 (33.9)	131 (31.8)	0.09	1.26 (0.96–1.64)
HWE			P = 0.256		

Table 4
Association of rs12617656 and rs12379034 genotypes with clinical manifestations of RA patients.

Characteristic	rs12379034 GG	rs12379034 GA	rs12379034 AA	*P* value
RF	44.25 ± 41.31	95.01 ± 114.09	76.61 ± 87.222	0.173
Anti-CCP	270.2 ± 329.81	146.1 ± 240.4	113.51 ± 198.31	0.093
DAS28	3.36 ± 0.81	2.71 ± 1.01	3 ± 1.29	0.129
ESR	16.45 ± 12.60	18.20 ± 17.44	18.30 ± 15.91	0.885
Characteristic	rs12617656 CC	rs12617656 CT	rs12617656 TT	*P* value
RF	93.76 ± 115.73	76.07 ± 88.62	82.21 ± 96.87	0.663
Anti-CCP	100.98 ± 120.28	124.21 ± 214.23	151.03 ± 250.65	0.468
DAS28	3.08 ± 1.16	2.97 ± 1.14	2.84 ± 1.21	0.612
ESR	19.78 ± 14.06	17.56 ± 13.61	19.12 ± 17.43	0.607

RA, Rheumatoid Arthritis; RF, Rheumatoid Factor; Anti-CCP, Anti-Cyclic Citrullinated Peptide Antibody; DAS28, Disease Activity Score for 28 Joints; ESR, Erythrocyte Sedimentation Rate.

frequently in RA patients compared with healthy individuals (33.9% *vs*. 31.88), in spite of insignificant difference (OR = 1.26, CI: 0.96–1.64; P = 0.09).

No significant correlations were observed between the genotypes of *DPP4* gene rs12617656 and *CDK5RAP2* gene rs12379034 SNPs and clinical data of RA patients (Table 4). Evaluated clinical data comprised of ESR (Erythrocyte sedimentation rate), DAS28 (Disease Activity Score in 28 Joints), RF (Rheumatoid factor), and anti-CCP (anti-Cyclic Citrullinated Peptide Antibody).

4. Discussion

Numerous studies have indicated that genetic factors play an important role and are responsible for about 60% of susceptibility and clinical outcomes of RA disease [15]. The association of RA with the *HLA-DRB1* gene is the well understood genetic association. However, many non-HLA loci have been associated with RA, such as the *TNFRSR11A* gene, which encodes the receptor activator of nuclear factor κB (RANK), playing an important role in bone resorption in RA [16]. On the other side, genetic factors are fruitful in the treatment of RA due to the activity of enzymes involved in the metabolism of drugs like azathioprine, methotrexate, methylenetetrahydrofolate reductase, and thiopurine methyltransferase, which are involved in modulation of genetic-caused manifestations [17,18].

DPP4 gene encodes DPP4 enzyme, also known as cell surface antigen CD26, which have serine protease activity and is involved in cleaving of a number of regulatory factors, such as growth factors and chemokines [19]. Agents with inhibitory action on DPP4 have recently been introduced as novel pharmacological compounds for treatment of inflammatory disease [20]. It has been shown that DPP4 was up-regulated in active RA patients [21]. DPP4 play an important role in T cell co-stimulation. The $CD4^+$ interleukin-17 producing T helper (Th) 17 cells play an important role in inflammation and seem to be involved in RA disease progression [22]. DPP4 is highly expressed on Th17 cells in comparison to other subsets of $CD4^+$ T lymphocyte. This molecule impresses on the chemotaxis of T cells through cleaving the proinflammatory chemokines such as CXCL9, CXCL10, CXCL11, and CXCL12 [23]. Association of *DDP4* gene rs12617656 SNP with increased risk of RA was first described in Han Chinese population. Furthermore, rs12617656 demonstrated a strong interaction effect with anti–citrullinated protein antibody (ACPA) [8]. Later, the association of this SNP was observed with increased risk of type 1 diabetes (T1D) in Malaysian population and related to serum DPP4 levels [24]. In this study, the *DPP4* gene rs12617656 SNP was evaluated in relation to RA risk in Iranian population. It was observed that rs12617656 polymorphism in *DPP4* gene was not a genetically contributing factor for RA susceptibility. The disparity in genetic association studies is common and usually attributed to differences in techniques, sample size, power of the study, ethnicity, and even environmental factors that individuals experience during life time.

First GWAS of RA patients in Han Chinese population demonstrated strong association of rs12379034 SNP, located in the coding region of *CDK5RAP2* gene, with susceptibility to the disease. Moreover, *CDK5RAP2* gene demonstrated connection with two genes, KIF23 and FGFR1OP, which already were associated with RA risk in European population [8]. CDK5RAP2 has been implicated to participate in the modulation of neuronal differentiation pathway [25]. However, no study has suggested that CDK5RAP2 might play role in the pathogenesis of autoimmune disorders, including RA. Nonetheless, the strong association of *CDK5RAP2* gene rs12379034 SNP with increased RA risk as well as its connection with other RA associated genes imply to new pathway in the pathogenesis of the disease. Investigation of possible connection between *CDK5RAP2* gene rs12379034 SNP in Iranian population revealed that this variant could not impress the risk of RA. However, further analyses of *CDK5RAP2* gene for other SNPs might contribute our understanding of novel pathogenesis pathway of RA with emphasis on neuronal differentiation perspective.

5. Conclusion

Considering all the facts, this was the first replicated study of *DPP4* gene rs12617656 and *CDK5RAP2* rs12379034 polymorphisms in Iranian RA population. None of these variants could impress the RA risk and could not be considered as the genetic contributing factor for the disease susceptibility. However, further investigations will be helpful in gaining clear knowledge of RA pathogenesis with respect of *DPP4* and *CDK5RAP2* genes.

Conflicts of interest

There is no conflict of interest to declare.

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