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Association study of CCR6 gene single nucleotide polymorphism with susceptibility to rheumatoid arthritis in Iranian population

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Several genome-wide association studies (GWASs) have identified numerous susceptibility genes for risk of rheumatoid arthritis (RA). Moreover, a bulk of the individual association studies in various populations has disclosed that genetics are significantly responsible for RA pathogenesis. CCR6 is a chemokine which is involved in the infiltration of inflammatory cells to sites of immune response. In this study, the association of *CCR6* gene rs1854853 single nucleotide polymorphism (SNP) with susceptibility to RA was evaluated in an Iranian population. The investigated population comprised 250 RA patients and 500 healthy individuals. Real time TaqMan MGB-based PCR allelic discrimination approach was employed to genotype the samples with regard to the *CCR6* gene rs1854853 SNP. Considering the A allele of rs1854853 SNP as reference, the G allele did not demonstrate a different prevalence between RA patients and controls (p= 0.17). Moreover, AG and GG genotypes were almost equally distributed between cases and controls (p= 0.61 and 0.14, respectively). Alternately, the dominant model of AG+GG had no significant difference in frequency between the study groups (p= 0.36). However, genotypes did show a correlation with the clinicopathological specifications of RA patients. Results suggest that the *CCR6* gene rs1854853 SNP is not involved in the genetic pathogenesis of RA in the Iranian population.

Keywords: *CCR6* gene, rheumatoid arthritis, single nucleotide polymorphism.

Introduction

Rheumatoid Arthritis (RA) is an obvious example of a disease encompassing definite features of rheumatologic manifestations with the signs of pain, swelling, and tenderness of joints which are caused by a mixture of autoimmune, genetic, and environmental factors [1, 2]. RA is characterized by synovium over-proliferation, cytokine and chemokine overproduction, autoantibodies such as anti-cyclic citrullinated peptide antibody (Anti-CCP) and rheumatoid factor (RF), angiogenesis, osteoclastogenesis, and cardiovascular and pulmonary complications [3]. In RA, several immune cells such as plasma cells, macrophages, dendritic cells (DCs), and lymphocytes as well as immune complexes infiltrate the synovium environment. Fibroblast-like synoviocytes in RA release various mediators like chemokines, cytokines, and matrix metalloproteinases (MMPs), which degrade the extracellular matrix and eradicate the articular structures in joints [4, 5].

To the best of the authors' knowledge and based on numerous studies performed in recent years, autoimmune responses along with genetic materials are the main culprits to be blamed for causing such diseases [6-9]. Genome-wide association studies (GWASs) have clarified new loci or genes that are linked to RA. In this regard, the more new genetic causalities are discovered, the more beneficial new approaches to cure the diseases can be found [10].

As a subset of $CD4^+$ T helper (Th) cells, IL-17-producing Th17 cells express CCR6 (CC chemokine receptor 6) on themselves, which is bound to its ligand (CCL20), eventuating in the homing of these cells to inflamed joints. By producing IL-17, a well-known inflammatory cytokine in most autoimmune diseases, Th17 cells play an important role in the pathogenesis of RA [11]. According to studies conducted on RA, some important risk alleles which impact the immune

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system have been revealed [12]. *CCR6* gene polymorphisms have been implicated in RA susceptibility [13, 14]. In addition to RA, *CCR6* gene SNPs have been associated with Crohn's disease, colorectal cancer, Graves' disease, and psoriasis [15-17]. Various SNPs in this gene, including rs3093024, rs3099023, and rs1854853, have been surveyed during recent years, especially in European, Southeast Asian, and African-American populations [18]. A meta-analysis indicated that the *CCR6* gene rs1854853 SNP was associated with an increased risk of RA in different populations [18]. Moreover, a GWAS reported the strong association of rs1854853 with the risk of RA in Han Chinese and European white populations as well as with the clinical outcomes of the disease [13].

Considering the important role of CCR6 in the immunopathogenesis of RA and previously-observed strong association of *CCR6* gene polymorphisms, particularly rs1854853, with the risk of RA, the current study evaluated the effect of this SNP on the risk of RA in an Iranian population. Furthermore, the association of genotypes of rs1854853 SNP with the clinical specifications of RA patients was analyzed.

Materials and Methods

Patients and Controls

From a total of 750 individuals, 250 RA patients (49 males and 201 females) who met 2010 American College of Rheumatology (ACR) classification criteria [19] and 500 healthy controls (113 males and 387 females) were recruited from the Outpatient Rheumatology Clinic of Shariati Hospital. None of the healthy control subjects had autoimmune or rheumatic diseases either in themselves or among their family members. The two groups were matched in terms of age, gender, and ethnicity. The mean age of patients was 50.91±12.60 and of controls was 42.49±12.97 years. All participants completed informed consent forms with total satisfaction. The study was approved by the Ethics Committee of Tehran University of Medical Sciences. To perform the experiments, 5 ml of peripheral blood was collected from each individual in EDTA-anticoagulated tubes and ESR blood collection test tubes using venipuncture.

Genotyping

Genomic DNA was extracted from peripheral blood using the phenol-chloroform method [20]. TaqMan MGB-based allelic discrimination technique was employed to genotype the subjects. PCR reaction mixture contained 4.5 µl of genomic DNA, 5 µl Taq-Man Master Mix containing Taq DNA polymerase and dNTPs (Applied Biosystems, Foster City, USA), 0.25µl 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 seconds, then 95 °C for 10 mins, subsequently 40 cycles of amplification (95 °C for 15 seconds and 60 °C for 1 min), and finally 60 °C for 30 seconds.

Statistical analysis

The chi-square test was utilized to analyze the polymorphism association with the disease. Odds ratio (OR) as the effect size with 95% confidence intervals (95% CI) were measured. Allele frequency and genotype distribution in the control group were evaluated to assess deviations from Hardy-Weinberg equilibrium (HWE). The association of clinical manifestations with the genotypes was also verified applying analysis of variance (ANOVA) using SPSS version 22 (SPSS Inc., Chicago, IL, USA). A $p< 0.05$ was considered as statistically significant.

Results

Table 1 shows the demographic features and laboratory findings of RA patients and healthy control groups. Autoantibodies, including RF and Anti-CCP, were positive in 175 (70.1%) and 160 (64%) RA patients, respectively. Deformity of the joints was seen in 45 (18%) patients. Moreover, morning stiffness and arthritis were observed in 120 (48%) and 140 (56%) patients, respectively. Measured values for the disease activity score-28 (DAS28), erythrocyte sedimentation rate (ESR), patient global assessment (PGA), visual analog scale (VAS), tender joint counts-28 (TJC28), and swollen joint counts-28 (SJC28) are shown in Table 1.

No significant deviation from the HWE ($p= 0.84$) was observed in the genotype distribution of the *CCR6* gene rs1854853 SNP in the healthy control population (Table 2).

The global major allele of A was assigned as the reference allele for rs1854853 SNP according to the NCBI database (https://www.ncbi.nlm.nih.gov/projects/SNP/). The frequency of the G allele for rs1854853 SNP was less in RA patients than controls (41.2% *vs*. 45.6%, respectively); however, the difference was not significant (OR= 0.84, CI: 0.65-1.08; $p= 0.17$). On the other side, the AA genotype was considered as the reference. The difference in the AG genotype frequencies was not significant between patient and

control groups (OR= 0.90, CI: 0.60-1.35; *p*= 0.61).

Table 1. Demographic data and laboratory specifications of rheumatoid arthritis patients and healthy controls

Characteristic	RA patients N=250 (%)	Healthy individuals N=500 (%)
Male	49 (19.6%)$^{+}$	113 (22.6%)
Female	201 (80.4%)$^{+}$	387 (77.4%)
Age	50.91±12.605*	42.49±12.971
Smoker	3 (1.2%)$^{+}$	42 (8.4%)
RF (positive)	175 (70.1%)$^{+}$	-
Anti-CCP (positive)	160 (64%)$^{+}$	-
DAS28	3.33±1.212*	-
ESR	19.87±17.10*	-
Deformity (positive)	45 (18%)$^{+}$	-
Morning stiffness (positive)	120 (48%)$^{+}$	-
Arthritis (positive)	140 (56%)$^{+}$	-
PGA	28.27±11.97*	-
VAS	28.18±11.87*	-
TJC28	2.89±3.42*	-
SJC28	2.13±3.18*	-

RA, Rheumatoid Arthritis; RF, Rheumatoid Factor; Anti-CCP, Anti-Cyclic Citrullinated Peptide Antibody; DAS28, Disease Activity Score for 28 Joints; ESR, Erythrocyte Sedimentation Rate, PGA, Patient global assessment; VAS, Visual Analogue Scale; TJC, Tender joint count; SJC, Swollen joint count.

* Data are presented as Mean ± Standard Deviation; $^{+}$ Data are presented as N (%).

Table 2. Allele and genotype distribution of *CCR6* rs1854853 SNP in RA patients and healthy controls

SNP	Allele /genotype	RA (N=250) N (%)	Control (N=500) N (%)	OR (95% CI)	*p* value
rs1854853	A	294 (58.8%)	544 (54.4%)	Reference	-
	G	206 (41.2%)	456 (45.6%)	0.836 (0.647-1.081)	0.17
	AA	82 (32.8%)	143 (28.6%)	Reference	-
	AG	130 (52%)	256 (51.2%)	0.90 (0.598-1.353)	0.61
	GG	38 (15.2%)	101 (20.2%)	0.66 (0.387-1.146)	0.14
	AG+GG	168 (67.2%)	357 (71.4%)	0.834 (0.565-1.230)	0.36
HWE		-	*p*= 0.84		

RA, Rheumatoid Arthritis; HWE, Hardy–Weinberg Equilibrium

Although the GG genotype was less frequent in RA patients than controls (15.2% *vs*. 20.2%, respectively), the difference was not statistically significant (OR= 0.66, CI: 0.39-1.15; *p* = 0.14). The AG+GG pattern was the dominant genotype, and it was represented more frequently in controls than in RA patients (71.4% *vs*. 67.2%, respectively); nonetheless, the distribution difference was not significant (OR= 0.83, CI: 0.56-1.23; *p* = 0.36).

The association of clinical manifestations of RA patients according to genotypes of *CCR6* gene rs1854853 revealed that the clinical values were not related to variations in genotypes (Table 3). The clinical items were RF, Anti-CCP, DAS28, ESR, deformity of joints, morning stiffness, arthritis, PGA, VAS, TJC28, and SJC28, and none of them were associated with rs1854853 GG, AG, or AA genotypes.

Discussion

The role of genetics in the onset or development of various types of disease, including autoimmune ones, has been one of the most controversial issues discussed in recently written articles. Researchers all over the world search for probable causal pathogenesis mechanisms in RA. Numerous studies have uncovered new loci in HLA or outside HLA regions that are

highly related to RA susceptibility, progression, or response to drugs such as disease-modifying antirheumatic drugs (DMARDs) [6, 21].

Table 3. Association of *CCR6* gene rs1854853 genotypes with various clinical features of the 250 RA patients

Characteristic	GG	AG	AA	*p* value
RF*	63 (36.8%)	68 (39.1%)	53 (30.5%)	0.613
Anti-CCP*	19 (50%)	77 (59.2%)	64 (78%)	0.293
DAS28	3.43 ± 1.25	3.22 ± 1.13	3.45 ± 1.31	0.409
ESR	23.62 ± 24.59	18.33 ± 14.21	20.56 ± 17.08	0.282
Deformity*	4 (10.5%)	30 (23%)	12 (14.6%)	0.146
Morning stiffness*	23 (60.5%)	59 (45.4%)	44 (53.6%)	0.365
Arthritis*	22 (57.9%)	66 (50.7%)	52 (63.4%)	0.241
PGA	28.1 3± 9.98	28.05 ± 12.19	28.70 ± 12.22	0.937
VAS	27.50 ± 9.837	28.23 ± 12.07	28.41 ± 12.55	0.936
TJC28	3.41 ± 3.38	2.60 ± 2.89	3.10 ± 4.14	0.412
SJC28	1.84 ± 2.23	2.04 ± 3.18	2.42 ± 3.55	0.631

RA, Rheumatoid Arthritis; RF, Rheumatoid Factor; Anti-CCP, Anti-Cyclic Citrullinated Peptide Antibody; DAS28, Disease Activity Score for 28 Joints; ESR, Erythrocyte Sedimentation Rate, PGA, Patient global assessment; VAS, Visual Analogue Scale; TJC, Tender joint count; SJC, Swollen joint count.

* Data are presented as number of positive (%); + Data are presented as number (%) in each cell.

RA is depicted as an autoimmune disease with systemic inflammation which specifically manifests as joint destruction and partial involvement of other organs [1]. Most patients experience intense inflammation with exacerbated health conditions over time, showing physical incapacitation and an over-representation of the mortality rate compared to the general population [3, 22-24]. Autoantibodies alongside cellular immunity (including $CD4^+$ T cells and related pro-inflammatory cytokines) which are prerequisites for progression of the disease constitute the main players of autoimmune diseases such as RA [3]. Based on recent studies, Th17 cells play a crucial role in RA progression by homing in on inflamed joints and mediating degradation of cartilage. The recruitment of Th17 cells to involved tissues occurs when CCR6 as a cell surface molecule is attached to its ligand, CCL20, which is produced in the synovial cells of RA patients [25]. CCR6 is also expressed on DCs, subsets of B Cells, and γδT cells which produce IL-17, contributing to the inflammation [14].

GWASs and studies of other associations have identified *CCR6* gene polymorphisms as a risk factor for RA predisposition. These studies have investigated populations with different ethnic backgrounds, however they represent contradictory results. Although most of them identified a significant relationship between polymorphisms and disease proneness, others did not observe any association [18]. The *CCR6* gene rs1854853 association was first discovered in a study on a Han Chinese population [13], as the G allele of this polymorphism was associated with the decreased risk of RA. Moreover, genotypes rs1854853 depicted a strong association with Anti-CCP-positive, but not with Anti-CCP-negative RA patients [13].

Based on our analysis, the current results are not in accord with some previous studies, and no significant association between *CCR6* gene rs1854853 polymorphism and RA risk in the Iranian population was observed. The inconsistency observed in gene association studies can be attributed to several issues. The first and most important one is deemed to be the racial or ethnic disparity between Iranians and other descents, explaining genetic heterogeneity. Another factor that should be considered is the difference in sample sizes among different studies.

In addition to the positive points of this study, such as the fairly large sample size and matching the case and controls with respect to ethnicity to eliminate population stratification bias, there were a number of limitations and caveats. The *CCR6* gene has been studied for a number of SNPs in autoimmune diseases. However, this study evaluated only one of them, and that may result in the omission of the concept of interactions between positions which function in an expanded network to cause RA. Moreover, the genetic SNPs of other genes in conjunction with the *CCR6* gene as regards immunological functions were not investigated.

Overall, this study investigated the association between the *CCR6* gene rs1854853 SNP and a predisposition to RA. No association was found

between alleles or genotypes of rs1854853 and RA risk. Furthermore, no association was observed between clinical features of RA patients and genotypes of rs1854853 SNP. It seems that the evaluation of this polymorphism in different ethnicities through comprehensive studies like meta-analyses will help researchers achieve a more valid understanding about the role of CCR6.

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Conflicts of interest

The author declares no conflicts of interest.

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