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RESEARCH ARTICLE

Association Analysis of IL-4 VNTR Polymorphism with Rheumatoid Arthritis in Iranian Patients

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ABSTRACT

Rheumatoid arthritis (RA) is a disorder characterized by movement disability and pain in the joints. This disease makes the body susceptible to other subsequent diseases, making the condition worse. To find out the underlying genetic diversity of this disease at the genomic level in an Iranian population, we carried out an investigation in the VNTR of IL-4 within its third intron. For this goal we isolated the genomic DNA from blood samples of 192 Rheumatoid arthritis patients and 182 healthy controls and investigated the presence or absence of specific amplicons via polymerase chain reaction (PCR). The size of each amplicon on a 1.5% agarose gel corresponded to a certain number of tandem repeats which indicated a specific allele. Statistical test of $\chi 2$ Fisher's exact test and odds ratio (OR) was used to analyze the data. The results showed that RA1/RA1 genotype was the dominant genotype in both healthy controls and patients and the heterozygote genotype of RA1/RA2 was observed more in the healthy controls than patients (36 vs 22) with significant difference of P value = 0.023 and odds ratio of 1.861. However two genotypes of RA2/RA2 and RA2/RA3 were exclusively observed in the patients' samples. We concluded that IL-4 VNTR polymorphism might have an association with rheumatoid arthritis and might be a high risk factor for development of Rheumatoid arthritis in the investigated Iranian population.
Keywords: IL-4, VNTR Polymorphism, Rheumatoid Arthritis, Iranian population

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by joint inflammation, pain and eventually movement limitation. 0.5-1.0% of adults are subject to rheumatoid arthritis in developed countries, with the incidence rate of 0.005-0.05% annually (1). During the course of Rheumatoid arthritis, the synovial tissues of the joints of hands, feet, and wrists become inflamed. In addition to articular symptoms, extra-articular manifestations can also occur. Some of these complications include: infection, malignancy, hematologic disorders, vasculitis, cardiac involvement (2), chronic obstructive pulmonary disease (3), reduced renal function (4), rheumatoid nodules (5), kerato-conjunctivitis sicca as the most frequent eye problem (6) and asthma (7). Both genetic predisposition and environmental factors play their role in initiating rheumatoid arthritis. It has been demonstrated that a shared amino acid motif in the HLA–DRB1 locus renders the carriers susceptible to rheumatoid arthritis via the auto-antibodies of rheumatoid factor and anti–citrullinated protein antibody (8).

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Cytokines have an inevitable role in the pathogenesis and autoimmunity in various phases of rheumatoid arthritis. The lack of balance between anti-inflammatory and pro-inflammatory cytokines is the main reason of inflammation, where higher levels of pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 overcome lower levels of anti-inflammatory cytokines like IL-4 and IL-13 (9). IL-4 along IL3, IL5, IL13 and CSF2 exist as a cytokine gene cluster on the long arm of chromosome 5 (NCBI gene ID: 3565). IL-4 as a strong anti-inflammatory cytokine, is produced by Th2 cells inhibits production of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, IL-8 and IL-12 (10) and LIF (11). It has been shown that systemic treatment with IL-4 significantly protects against cartilage destruction in mouse models (10). Supernatants from cultured synovium pieces from patients were analyzed and it was showed treatment with IL-4 reduced IL-1 beta and increased synthesis of IL-1 receptor antagonist, induced from monocytes and macrophages from RA synovium (12).

Many cytokines and their receptors demonstrate genetic polymorphisms mostly described as single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs) (13). The third intron of the IL-4 gene, contains a VNTR polymorphism with a size of 70 bp occurring at different repeats. The most common allelic form of IL-4 VNTR consists of three repeats (allele 1), while a less common allele with two repeats (allele 2), and a much rarer allele with four repeats (allele 3) do exist (14).

Materials and Methodologies

Patients and controls

In our study we took blood samples from 192 RA patients with chronic rheumatoid arthritis, all from Baqiyatallah hospital in Tehran according to patients' standard ethical guideline in year 2015 aged between 44 and 70. All the patients had the common symptoms of rheumatoid arthritis including knee and wrist swelling, suffering from the condition for at least 5 years. The control population consisted of 182 anonymous healthy blood donors from the city of Tehran with the same genetic background as the patients.

DNA analysis and genotyping

Blood samples were taken in 5 mL tubes containing Na-EDTA from both RA patients and healthy individuals. Genomic DNA was isolated from each blood sample of both patients and volunteered healthy individuals in the study. The genomic DNA was extracted from 2 ml of white blood cells (WBC) of peripheral uncoagulated blood sample by the salting-out method and stored at a final concentration of 20-50 ng/µl at 4° C for further genotyping. IL-4 VNTR has been reported as variable number of repeats in the intron 3 of IL-4 gene, and the size of the resulting amplicon is indicative of the number of repeats in the genomic sequence. The corresponding size and allele designation are as follows: Amplicon size of 342bp (Allele1) 272bp, (Allele2) and 412bp (Allele3).

Primer sequences used according to previous reports with PCR conditions of initial denaturation, 95°C for 2 min, followed by 38 cycles of 95°C for 1 min, 56°C for 1 min and final extension of 72° C for 5 min (14). The PCR reaction was carried out in total volume of 10 μL that consisted of primers with final concentration of 0.5μM (Synthesized by Sinaclon) and 2 mM MgCl2 Taq polymerase 2x Master Mix (Ampliqon) and 50 ng of genomic DNA. PCR products were run on 1.5% agarose gel by electrophoresis and visualized by the aid of SYBR Green I and compared to the 50bp DNA marker (Sinaclon).

Statistical analysis

In our study groups of control and patient, the differences between genotypes and allele frequencies were analyzed by SPSS 23.0 and P values were calculated according to $\chi 2$ Fisher's exact test for 2×2 tables and P values less than 0.05 were considered as statistically significant. The allele frequencies were calculated according to the Hardy-Weinberg equilibrium. The odds ratio (OR) with confidence intervals of 95% (95% CI) were also calculated.

Results

Genotypes and allele frequencies in patients and controls

The genotype and allele frequencies of IL-4 VNTR polymorphism were statistically analyzed in RA patients and controls (Table 1). The number of RA1/RA1 genotype and the frequency of RA1 allele was highest in both RA patients and controls groups. The difference between the number of RA1/RA2 genotypes in the control and healthy groups was significant (P= 0.023). The risk of RA was higher in individuals with RA1/RA1 genotype in comparison to individuals with RA1/RA2 genotype. The RA2/RA2 and RA2/RA3 genotypes were only seen in RA patients.

Table 1: Genotypes and allele frequency of the IL-4 VNTR polymorphism in RA patients and controls.

Genotype	Healthy Controls *N* = 182 (%)	RA Patients *N* = 192 (%)	P value	OR (95% CI)
RA1/RA1	146 (80%)	166 (86%)	Ref	
RA2/RA2	0 (0%)	2 (1%)	0.285	0.988 (0.972-1.005)
RA1/RA2	36 (20%)	22 (11%)	0.02	1.861 (1.047-3.307)
RA2/RA3	0 (0%)	2 (1%)	0.285	0.988 (0.972-1.005)
Allele frequency				
RA1	330 (90.16%)	354 (92.19%)	Ref	
RA2	36 (9.84 %)	28 (7.29 %)	0.137	1.028 (0.984-1.074)
RA3	0 (0 %)	2 (0.52 %)	0.269	0.994 (0.987-1.002)

Discussion

In this study, we analyzed the association between VNTR polymorphism and development of RA of IL4 in a group of Iranian population to find out which genotypes could be a risk factor for the condition. We found an association between intron 3 VNTR polymorphism of IL4 gene and RA in our population of study. In our study the control group only showed two genotypes of RA1/RA1 and RA1/RA2 but the patients' genotype exclusively showed RA2/RA2, RA2/RA3 genotypes in addition to the genotypes observed in the control group, however RA1/RA3 and RA3/RA3 genotype was absent in both groups.

Previously various genes with SNPs have been marked in association with the pathogenesis of rheumatoid arthritis, including HLA-DRB1, PTPN22, PADI4, STAT4, FCGR2A, CTLA-4, CCL21, TRAF1, IRF5, CCR6, CD40, IL2RA, TNF α and interleukins (15). Interleukins as a large family of cytokines have been thoroughly investigated in regard to gene SNPs studies in RA patients. It has been shown that carriage of the rare allele 2 of IL-1B was associated with destructive arthritis as compared to non-destructive arthritis (16). In addition to the intron region, it has been reported that two polymorphisms in the promoter region of IL-6, provides a strong susceptibility for European RA patients compared to Asians (17). VNTR polymorphisms have been shown different repeats in introns or exons of various genes including interleukins. A VNTR polymorphism in the intron 2 of interleukin-1 receptor antagonist (IL-1RN) revealed five different alleles and like IL-4 VNTR polymorphism allele 2 was associated with systemic lupus erythematosus (18).

Many studies have shown the association of VNTR polymorphism of the IL4 gene in various populations including immunologic diseases of rheumatoid arthritis (19), periodontitis (20), type-2 diabetes (21), systemic lupus erythematosus (22), vitiligo (23), multiple sclerosis (24), end-stage renal disease (25), carcinoma of the urinary bladder (26), alopecia areata (27) and recurrent aphthous stomatitis, where VNTR polymorphism in intron 3 of IL4 gene showed statistically different values between patients and control group, and allele 2 was protective against this condition (28). In a study of IL-4 VNTR polymorphism on preeclampsia, another immune related disease, the homozygote genotype for allele 2 was only demonstrated for patients, however in conflict with our study the homozygote genotype for allele 2 was only observed in healthy controls and the heterozygote genotype of RA1/RA2 was observed higher in the patients' samples (29) while that was opposite in our study In our study and the carriage of the rare allele 2 of IL-4 was higher in the control group and the carriage of rarest allele 3 was exclusively shown in two patients' samples. We also demonstrated that RA1/RA1 genotype was the dominant genotype and RA1 allele was the most frequent one in both study groups. In conclusion we demonstrated IL-4 VNTR polymorphism might be a

high risk factor for development of RA in the Iranian population.

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