ISSN 1022-7954, Russian Journal of Genetics, 2017, Vol. 53, No. 3, pp. 406–409. © Pleiades Publishing, Inc., 2017.

SHORT COMMUNICATIONS

# Association Analysis of IL-4 VNTR Polymorphism with Rheumatoid Arthritis in Iranian Patients[1]

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Received May 30, 2016

**Abstract**—Rheumatoid arthritis (RA) is an autoimmune disease characterized by movement disability and pain in the joints. The affected individuals are susceptible to other subsequent diseases, exacerbating the condition. To find out the genetic variability of this disease at the genomic level for the first time in the Iranian population, we carried out an investigation on the VNTR of IL-4 gene within its third intron. For this goal we isolated the genomic DNA from blood samples of 576 rheumatoid arthritis patients and 546 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 (108 vs. 66) with significant difference with $P$ value < 0.005 and odds ratio of 0.214. However two genotypes of RA2/RA2 and RA2/RA3 were exclusively observed in the patients' samples with $P$ value = 0.023 and odds ratio of 0.988. We concluded that IL-4 VNTR polymorphism has a strong 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 patients

**DOI:** 10.1134/S1022795417030036

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by joint inflammation, pain and eventually movement limitation. Approximately 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]. 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 with 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 [10] and one of 5 common key players in all allergic diseases [11] is produced by Th2 cells and inhibits production of pro-inflammatory cytokines such as IL-1, TNF-α, IL-6, IL-8 and IL-12 [10] and LIF [12]. 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 [13].

Many cytokines and their receptors demonstrate genetic polymorphisms mostly described as single

[1] The article is published in the original.
Supplementary materials are available for this article at DOI: 10.1134/S1022795417030036 and are accessible for authorized users.

Calculated genotype and allele frequency for IL-4 VNTR polymorphism in RA patients and controls

| Genotype, allele | Healthy controls | RA patients | *P* value | OR (95% CI) |
|---|---|---|---|---|
| Genotype | *N* = 546 | *N* = 576 | | |
| RA1/RA1 | 438 (80%) | 498 (86%) | Ref | |
| RA2/RA2 | 0 (0%) | 6 (1%) | 0.023 | 0.988 (0.979–0.998) |
| RA1/RA2 | 108 (20%) | 66 (11%) | <0.0005 | 0.214 (0.137–0.333) |
| RA2/RA3 | 0 (0%) | 6 (1%) | 0.023 | 0.988 (0.979–0.998) |
| Allele | *N* = 1098 | *N* = 1152 | | |
| RA1 | 990 (90.16%) | 1062 (92.19%) | Ref | |
| RA2 | 108 (9.84%) | 84 (7.29%) | 0.020 | 0.745 (0.567–0.979) |
| RA3 | 0 (0%) | 6 (0.52%) | 0.019 | 0.994 (0.990–0.999) |

nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs) in various geographic locations [14, 15]. Functional role of VNTR polymorphism has been demonstrated in human where its effect is determined by the number of tandem repeats [16]. The third intron of the IL-4 gene, contains a VNTR polymorphism with a size of 70 bp occurring as 2 to 4 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 [17].

*Patients and controls.* In our study we took blood samples from 576 RA patients with chronic rheumatoid arthritis that were admitted to Baqiyatallah hospital in Tehran according to patients' standard ethical guideline in 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 546 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 343 bp (allele 1), 272 bp (allele 2) and 412 bp (allele 3). PCR was carried out according to a previous report [17] with 38 cycle repeats.

The PCR reaction was carried out in total volume of 10 μL that consisted of primers with final concentration of 0.5 μM (synthesized by Sinaclon) and 2 mM $MgCl_2$ *Taq* polymerase 2× 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 (Bio-Rad) and compared to the 50 bp DNA marker (Sinaclon).

*Statistical analysis.* The data obtained from groups of control and patients and 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 odds ratio (OR) with confidence intervals of 95% (95% CI) were also calculated. The allele frequencies were calculated according to the Hardy–Weinberg equilibrium.

Due to the specificity of the primers the molecular size of each amplicon corresponded to a certain allele. The most common allele was allele 1 and the rarest allele was allele 3.

*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). The number of RA1/RA1 genotype and the frequency of RA1 allele the reference 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 very significant with *P* value < 0.005 showing more RA1/RA2 genotype in the healthy controls. The risk of RA was higher in individuals with RA1/RA1 genotype in comparison to individuals with other genotypes. The RA2/RA2 and RA2/RA3 genotypes were only seen in RA patients both with *P* value = 0.023 and OR 0.988 (0.979–0.998).

In this study, we analyzed the association between IL-4 VNTR polymorphism and development of RA for the first time in a group of Iranian population to find out which genotypes could be a risk factor for this condition. We found an association between intron 3 VNTR polymorphism of IL-4 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 observed control group genotypes, however RA1/RA3 and RA3/RA3 genotypes were absent in both groups.

Several other genes with SNPs have been reported in association with the pathogenesis of rheumatoid arthritis, including HLA-DRB1, PTPN22, PADI4, STAT4, FCGR2A, CTLA-4, CCL21, TRAF1, IRF5, CCR6, CD40, IL2RA, TNFα and other interleukins [18]. Interleukins as a large family of cytokines have been thoroughly investigated in regard to SNP 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 [19]. 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 [20]. 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 [21]. Many studies have shown the association of VNTR polymorphism of the IL-4 gene in various populations, including immunologic diseases of rheumatoid arthritis [22], periodontitis [23], type-2 diabetes [24], systemic lupus erythematosus [25], vitiligo [26], multiple sclerosis [27], atopic asthma [28] end-stage renal disease [29], carcinoma of the urinary bladder [30], alopecia areata [31] and recurrent aphthous stomatitis [32] where VNTR polymorphism in intron 3 of IL-4 gene showed statistically different values between patients and control group. In a study investigating IL-4 VNTR polymorphism on pre-eclampsia—another immune related disorder—in Iraninan patients, the homozygote genotype for allele 2 (RP2/RP2) was only observed in patients' samples, that was concurrent with our study for allele 2 (RA2/RA2), which was only observed in patients with rheumatoid arthritis. However on the contrary the heterozygote genotype of RP1/RP1 was observed higher in control samples which we observed higher in patients' samples and RP1/RP2 genotype was observed higher in the patients' samples, which we observed higher in control samples [33]. However, we distinguishably demonstrated the existence of the RA2/RA3 genotype exclusively in the patients' samples.

Unfortunately due to some limitations we could not correlate the genotypes with plasma levels of IL-4, however a study reported that patients with RA2/RA2 genotype in regard to IL-4 VNTR had lower levels of plasma concentration of IL-4 compare to RA1/RA1 and RA1/RA2 genotypes [34], which partly explains why this genotype was observed only in our patients's samples. We 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 that IL-4 VNTR polymorphism might indicate a risk factor for development of RA in the Iranian population.

## ACKNOWLEDGMENTS

I appreciate the cooperation of the hospital and genetic research center of Baqiyatallah University of Medical Sciences for funding and providing all the required facilities. I would like to thank Mr. Mostafa Hosseini for his assistance in ratification of the proposal and also thank Mr. Iman Mortazvi for his assistance in 01 Lab and all the governmental authorities granting this opportunity to carry out this research as part of the national civil service.

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## Supplementary Information

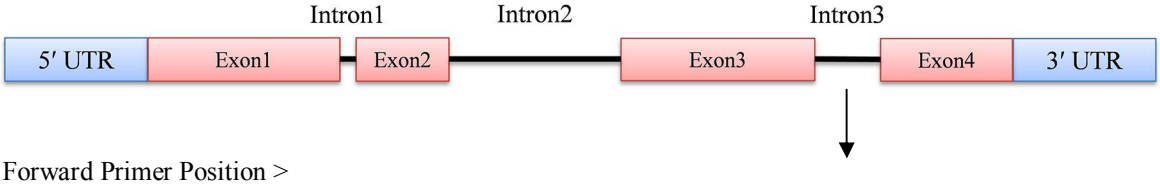
"Russian Journal of Genetics" v. 32, no. 3, 2016.

Supplementary table and figure to the manuscript "Association Analysis of IL-4 VNTR Polymorphism with Rheumatoid Arthritis in Iranian Patients" A. Beh-Pajooh, M. Fasihi-Ramandi, M. Tavallaie.

**S1.** PCR conditions for amplification of IL-4 VNTR alleles. (Ref: No.17)

| **Program** | **Temperature** | **Duration** |
|---|---|---|
| Initial Denaturation | 95°C | 2 min |
| Denaturation | 95°C | 1 min |
| Annealing | 56°C | 1 min |
| Extension | 72°C | 30 Sec |
| Final Extension | 72°C | 5 min |

**S2.** Overview of IL-4 gene Allele 1 and three times repeat of the 70bp VNTR sequence within Intron 3. (Ref: Ensembl Transcript: IL4-001 ENST00000231449.6)




Forward Primer Position >

GTAAATAGGCTGAAAGGGGGAAAGCATAGAAGCAAGATGGCCTGTTGGGAGGCTACCACAGTAAACCAGG
CATTTATCCGACTTTCCCCCTTTCGTATCTTCGTTCTACCGGACAACCCTCCGATGGTGTCATTTGGTCC

CTAGAGATGATGGTGGCGTGGACAGAATGAAGCAAGATGGCCTGTTGGGAGGCTACCACAGTAAACCAGG
GATCTCTACTACCACCGCACCTGTCTTACTTCGTTCTACCGGACAACCCTCCGATGGTGTCATTTGGTCC

CTAGAGATGATGGTGGCGTGGACAGAATGAAGCAAGATGGCCTGTTGGGAGGCTACCACAGTAAACCAGG
GATCTCTACTACCACCGCACCTGTCTTACTTCGTTCTACCGGACAACCCTCCGATGGTGTCATTTGGTCC

CTAGAGATGATGGTGGCGTGGACAGAATGGGAGCAGTTGAGGTGAACAGATTTGGGATATGACTAAAAAT
GATCTCTACTACCACCGCACCTGTCTTACCCTCGTCAACTCCACTTGTCTAAACCCTATACTGATTTTTA

AAAACCAGAAGGATTTGCTGACAGATCGGTTGTAGGGGGTAAGATACAGGGGAGGAAAAGATGACCTCTT
TTTTGGTCTTCCTAAACGACTGTCTAGCCAACATCCCCCATTCTATGTCCCCTCCTTTTCTACTGGAGAA

< Reverse Primer Position