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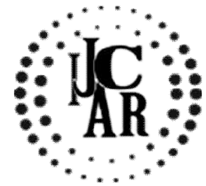

Research Article

ASSOCIATION STUDY OF IL-1RA GENE (INTRON 2 -86 bp) VNTR POLYMORPHISM WITH RHEUMATOID ARTHRITIS IN VINDHYAN POPULATION

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A B S T R A C T

Rheumatoid arthritis (RA) is a chronic, systemicdisease of unknown etiology. Several studies have reported avariable number of tandem repeat (VNTR) 86 bp (rs2234663) in the intron 2 of IL1RN gene with RA risk. The present study was designed to determine the frequencies of this polymorphism in patients with RA and control subjects (CS) and its association with RA in a western Mexican population. An analytical cross-sectional study was performed, in which 350patients with RA and 307 CS were included. The identification of IL1RN VNTR polymorphism was carried out by polymerase chain reaction (PCR), and genotypes were associated with clinical variables (DAS28 and CRP).
Polymorphism of the IL-1Ra gene, displays different copies of 86-bp tandem repeat in intron 2. The number of times this sequence is referred to as the copy number of the 86-bp sequence which in turn corresponds to different alleles of this IL-1Ra polymorphism. Five alleles of IL-1RA 86-bp VNTR polymorphism are as follows were classified on basis of number of repeats of 86 bp sequence and PCR product size on gel and designated as A1 (410 bp, 4 repeats), A2 (240 bp, 2 repeats), A3 (500 bp, 5 repeats), A4 (325 bp, 3 repeats) and A5 (595 bp, 6 repeats).Genotype frequencies, allele frequencies and carriage rates of IL-1Ra VNTR alleles are depicted in table no. 4.6. Genotype frequency between rheumatoid arthritis patient and healthy control groups were slightly different and significantly associated with arthritis ($\chi 2$=15.25 P=0.0184*). Thus allele frequency ($\chi 2$=15.44 P=0.0015**) and carriage rate ($\chi 2$=12.60, P=0.0056**) were also significantly different between both case and control groups.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic disease of unknown etiology, characterized by the production of proinflammatorycytokines and autoantibodies which lead to inflammation of the synovial membrane and subsequent destruction of cartilage and bone. This pathology is considered as multifactorial because hormonal, environmental, and genetic factors have been implicated. The genetic factor is the most important in the development of RA; genes associated with the major his to compatibility complex (MHC) may confer susceptibility to 30%. However, there are many other genes non-associated to MHC implicated in RA**[2].** New genetic markers have been investigated to help the early detection and classification of disease in different populations. Several studies have reported gene polymorphisms in theinterleukin-1 family associated with RA susceptibility such asa variable number of tandem repeat (VNTR) 86 bp(rs2234663) in the intron 2 of the interleukin-1 receptor antagonist(IL-1Ra) gen. IL-1Ra is considered as an antiinflammatorycytokine that inhibits the pro-inflammatory effects of interleukin-1 beta (IL-1β); therefore, it contributes in decreasing inflammatory process**[1-3].**

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DNA sequences of the human genome reveal that many genes are polymorphic. In coding or noncoding regions of as pecific gene, there may be either a single base pair substitution of one nucleotide for another (SNPs) or avariable number of repeats of a short repetitive DNA sequence (VNTR). Gene-environment interactions may be manifested in various ways, either by risk effects based on an individual's genotype, or differential gene risk effects based on exposure. The study of genetic polymorphisms promises to help definepathophysiological mechanisms, to identify individuals atrisk for disease and to suggest novel targets for drugtreatment. Cytokines are signaling molecules contributing to the inflammatory response, and are key components in the pathogenesis of many diseases like cancer, metabolicdisorders and inflammatory conditions**[5].** Interleukin-1β, located at chromosome 2q12, is apotent proinflammatory agent that is central inimmunoregulation, inflammation and cancer formation. IL-1 receptor antagonist (IL-Ra), astructural variant of IL-1, binds to the same IL-1 receptorand acts as a competitive inhibitor of IL-1 bioactivity. In the second intron of the IL-1RN gene, there is a functional VNTR polymorphism, which is characterized as having an important role in regulating the serum IL-Ra levels, humanimmune

response and cancer risk. Interleukin (IL)-1 is known to act as a tumor growth factor by inducing angiogenicfactors **[6].** IL1 family plays an important role in the destruction of articularcartilage, by decreasing synthesis of matrix components and increasing synthesis of matrix metalloproteinases. There are 11members of the IL-1 family of cytokines and 10 members of the IL-1family of receptors.IL1 receptor antagonist (IL1-RA) is an important anti-inflammatory molecule, which can bind to IL1 receptors in competition with IL1a and IL1b, thus inhibit their activities and modulate a variety of IL1-related immune and inflammatory activities. The IL1-RA gene (IL1-RN) has a variable number of tandem repeats (VNTR) polymorphism of 86 base pairs (bp) in intron 2. There are five alleles, corresponding to allele 1(four repeats), allele 2 (two repeats), allele 3 (five repeats), allele 4(three repeats) and allele 5 (six repeats), which can be further summed up as a short allele (2:2 repeats) and a long allele (S:3e6repeats)**[4-8].**

MATERIALS AND METHODS

Patient recruitment

Rheumatoid arthritis patients were recruited from sanjay Gandhi hospital, Rewa, Hamidia hospital, Bhopal; Bombay hospital Indore, (M.P.) during the year 2020-2021. 112 patients were enrolled in the study. All the patients were of Central Indian origin. The dignosis of RA was based on various laboratory tests (Rheumatoid Factor, Sed rate, Hemocrit, Synovial fluid analysis, Citrulline antibody, Antinuclear antibodies (ANA), C-Reactive Protein(CRP), Anti-CCP antibodies) and radiological criteria. All patients participating in study provided informed consent. Institutional ethics committee of Shyam Shah medical college, Rewa (M.P.), India, approved the experimental protocol.

Sample collection

112 randomly selected healthy controls (HC) were enrolled in the study. They consisted of medical staff and healthy volunteers from Rewa, Jabalpur, Bhopal, Indore as well as individuals residing in central region of India. Hence, control group was drawn from same area assuming similar environmental and social factors. Approximately 5 ml. of blood sample was collected in 0.5 M EDTA tubes from each RA patients as well as from healthy controls. These samples were stored frozen at -80˚C until DNA was extracted from them.

Method for DNA isolation

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frogen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was then allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nuceic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transfering of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantitating.

Determination of quality and quantity of isolated DNA

The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size heterogeneity is among the most important criterion for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

Quantitation by UV spectrophotometry

The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA prepration was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

Agarose Gel Electrophoresis

Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA prepration appears as single band. A horizontal agarose slab gel electrophoresis apparatus (BangloreGenei, Bangaore, India) was used. In brief, 2 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solutions (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco* RI / *Hind* Ш double digest (BangloreGenei, Bangaore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (VilberLourmate, Cedex 1, France) respectively.

Polymorphism screening

In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest. All the PCRs were carried out in a PTC 200 thermocycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative controle was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative controle used for PCR contained whole PCR reaction mix except target DNA which was replased by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called "star activity" which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO). The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5µg/ml) and subsequently visualized and photographed under UV transilluminator.

Detection of interleukin-1 receptor antagonist (IL1-Ra) 86 bp VNTR DNA polymorphism

The intron 2 of IL-1Ra gene contains a VNTR of 86 bp length of DNA. This region was amplified using PCR. The alleles were designated according to their respective base pair size taking the 86 bp core repeat sequence of IL-1Ra VNTR marker from literature (Tarlow *et al*., 1993). Five alleles were observed corresponding to the length variation in different copy numbers of the 86 bp sequence. They were designated as follows: Allele 1(410 bp, four repeats of 86 bp sequence), Allele 2 (240 bp, two repeats of 86 bp sequence), Allele 3 (500bp, five repeats of 86 bp sequence), Allele 4 (325 bp, three repeats of 86 bp sequence), Allele 5 (595 bp, six repeats of 86 bp sequence).

Primers

The oligonucleotides (primers) used were the sequences flanking this region described by Tarlow *et al*. (Tarlow *et al*., 1993). They are as follows:
IL1-Ra forward primer - 5' CTCAGCAACACTCCTAT 3'
IL1-Ra reverses primer - 5' TCCTGGTCTGCAGGTAAT 3'

PCR Mix

25 µl of each PCR reaction mixture contained 2-5 µl template DNA (final concentration 100-200 ng/ µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd., India), 1 µl of 10 mM dNTPs (BangloreGenei, Bangalore, India), 1 µl of 10 pm/µl of forward and reverse primers specific for IL-1Ra gene, 0.3 µl of 5U/ µl of *Taq* DNA polymerase (final concentration 1.5U; BangloreGenei, Bangalore, India) and sterile water to set up the volume of reaction mixture to 25 µl.

Thermal Profile

Thermal profile used for the amplification of desired segment of gene was as follows: Initial denaturation at 95˚C for 5 min and 30 cycles of denaturation at 94˚C for 1 min, annealing at 58˚C for 1 min and extention at 72˚C for 1 min, followed by final extension at 72˚C for 5 min. PCR products were separated on 2% agarose gel (2% w/v, Sigma) using a 100 bp molecular weight (MW) marker to confirm different PCR product sizes indicating presence of different alleles of VNTR.

Genotyping

15 possible genotypes were expected depending upon the PCR pattern. They are as follows : Genotype 1/1(410 bp), genotype 1/2 (410 bp and 240 bp), Genotype 1/3 (410 bp and 500 bp), Genotype 1/4 (410 bp and 325 bp), Genotype 1/5 (410 bp and 595 bp), Genotype 2/2 (240 bp), Genotype 2/3 (240 bp and 500 bp), Genotype 2/4 (240 bp and 325 bp), Genotype 2/5 (240 bp and 595 bp), Genotype 3/3 (500 bp), Genotype 3/4 (500 bp and 325 bp), Genotype 3/5 (500 bp and 595 bp), Genotype 4/4 (325 bp), Genotype 4/5 (325 bp and 595 bp), Genotype 5/5 (595 bp).

RESULTS

Detection of IL-1Ra intron 2 -86 bp VNTR polymorphism

The genes encoding for IL-1Ra are located on the long arm of chromosome 2 in humans (2q13-14). Polymorphism of the IL-1Ra gene, displays different copies of 86-bp tandem repeat in intron 2. The number of times this sequence is referred to as the copy number of the 86-bp sequence which in turn corresponds to different alleles of this IL-1Ra polymorphism. Five alleles of IL-1RA 86-bp VNTR polymorphism are as follows were classified on basis of number of repeats of 86 bp sequence and PCR product size on gel and designated as A1 (410 bp, 4 repeats), A2 (240 bp, 2 repeats), A3 (500 bp, 5 repeats), A4 (325 bp, 3 repeats) and A5 (595 bp, 6 repeats).

All the samples of RA group and HC group were genotyped using PCR for IL-1RA 86-bp VNTR polymorphism using the primers flanking the 86 bp length of DNA of intron 2 of IL-1Ra gene. The PCR products were analyzed on 2% gel along with 100 bp MW marker to confirm the expected product size of each allele present in each sample of patients and control

group. In our population we found four alleles (A1, A2, A3 and A4) and 7 genotype (Depicted in figure no. 1.)

Genotype frequencies, allele frequencies and carriage rates of IL-1Ra VNTR alleles are depicted in table no. 4.6. Genotype frequency between rheumatoid arthritic patient and healthy control groups were slightly different and significantly associated with arthritis ($\chi2$=15.25 P=0.0184*). Thus allele frequency ($\chi2$=15.44 P=0.0015**) and carriage rate ($\chi2$=12.60, P=0.0056**) were also significantly different between both case and control groups.

Odds ratio of A1/A1 genotype was indicate protective effects to disease thus A1 (P=0.0033**) allele are also protective allele.

to controls whereas genotype A2/A2 and A1/A2 were significantly increased in RA compared to HC group. An odds ratio of when compared to RA respectively indicated the protective effect of this genotype. Allele 2 showed a significant increase frequency and carriage rate in disease group as compared to controls. On the contrary allele A1 showed the high frequency and increased carriage rate (75.00%) in control population. Genotype A1/A3 was found no significant differences in distribution of this genotype among control population and RA population (03.12 % vs 0.89%) respectively. Genotype A2/A3 was no significantly distributed in HC group as compared to RA group (04.16% vs 0.89 %). Allele A4 (06.25% vs 0.89%) was also found in case and control population **(See Table No. 4.6 and 4.7).**

Table no 1 Frequency distribution and association of Genotype, allele frequency and carriage rate of IL-1Ra gene polymorphism in population of Vindhyan region using Chi Square Test

IL-1Ra GENE	CASE N= 96		CONTROL N=112		CHI SQUARE VALUE χ^2(P Value)
	N	%	N	%	
Genotype					
A1/A1	51	53.12	78	69.64	
A1/A2	23	23.95	28	25.00	
A1/A3	03	03.12	01	0.89	
A1/A4	08	08.33	01	0.89	
A2/A2	03	03.12	02	01.78	15.25 (0.0184*)
A2/A3	04	04.16	01	0.89	
A2/A4	04	04.16	01	0.89	
Allele					
A1	136	70.83	186	83.03	
A2	37	19.27	34	15.17	
A3	07	03.64	02	0.89	15.44 (0.0015**)
A4	12	06.25	02	0.89	
Carriage Rate					
A1	85	61.69	108	75.00	
A2	34	24.63	32	22.22	
A3	07	05.07	02	01.38	12.60 (0.0056**)
A4	12	08..69	02	21.92	

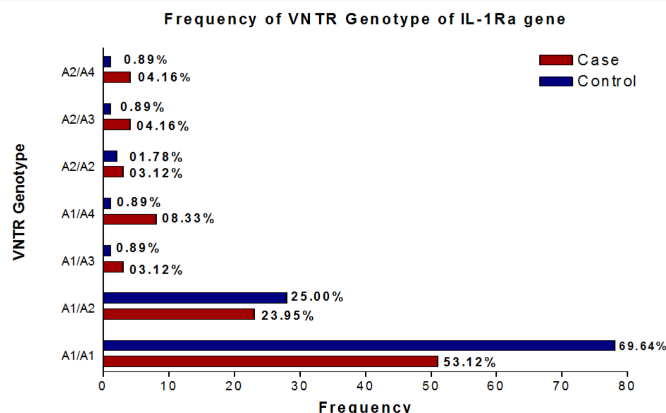
(* - denotes the level of significant association between case and control.)
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.

Table No 2 Fisher Exact Test values of IL-1Ra gene polymorphism

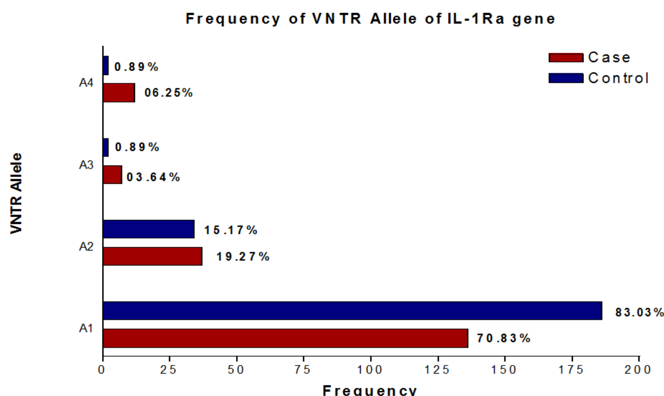
IL-1Ra GENE	CASE N= 96		CONTROL N=112		P Value	Odds Ratio (95% confidence interval)
	N	%	N	%		
Genotype						
A1/A1	51	53.12	78	69.64	0.0155*	0.4940 (0.2798 to 0.8722)
A1/A2	23	23.95	28	25.00	0.8732 ns	0.9452 (0.5011 to 1.783)
A1/A3	03	03.12	01	0.89	0.3371ns	3.581 (0.3661 to 35.02)
A1/A4	08	08.33	01	0.89	0.0128*	10.09 (1.238 to 82.25)
A2/A2	03	03.12	02	01.78	0.6627ns	1.793 (0.2932 to 10.97)
A2/A3	04	04.16	01	0.89	0.1835 ns	4.826 (0.5298 to 43.96)
A2/A4	04	04.16	01	0.89	0.1835 ns	4.826 (0.5298 to 43.96)
Allele						
A1	136	70.83	186	83.03	0.0033**	0.4962 (0.3108 to 0.7920)
A2	37	19.27	34	15.17	0.2967 ns	1.334 (0.7996 to 2.225)
A3	07	03.64	02	0.89	0.0873 ns	4.200 (0.8617 to 20.47)
A4	12	06.25	02	0.89	0.0044 **	7.400 (1.634 to 33.50)
Carriage Rate						
A1	85	61.69	108	75.00	0.0208 *	0.5346 (0.3210 to 0.8902)
A2	34	24.63	32	22.22	0.6742 ns	1.144 (0.6590 to 1.987)
A3	07	05.07	02	01.38	0.0975ns	3.794 (0.7739 to 18.60)
A4	12	08..69	02	21.92	0.0052**	6.762 (1.484 to 30.81)

(* - denotes the level of significant association between case and control.)
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)

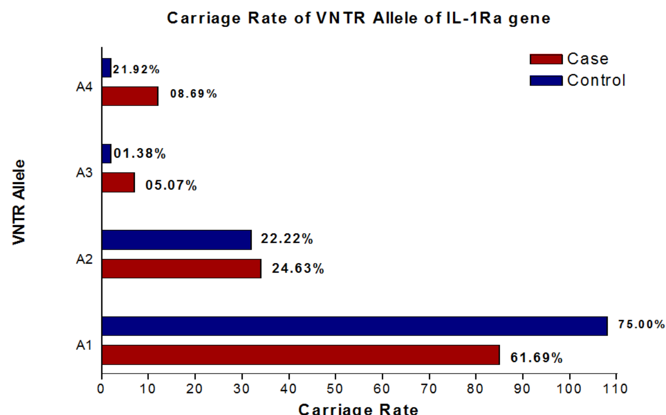
The genotype A1/A1 (P=0.0155*) and A1/A4 (P=0.0128*) were significantly associated with arthritis. A1/A1 genotype showed an overall significant difference in both groups with high odds ratio 0.4940 in RA group respectively as compared



Graph No 1 Genotype Frequency of IL-1Ra gene



Graph No 2 Allele Frequency of IL-1Ra gene



Graph No 3 Carriage rate of IL-1Ra gene

DISCUSSION

Interleukin-1 receptor antagonist (IL1-RN) gene was a Variable Number of Tandem Repeats (VNTR) polymorphism on the risk of RA. The strength of the association between the IL1-RN VNTR polymorphism and the risk of RA was assessed by odds ratios (ORs) with the corresponding 95% confidence interval (CI) for each study. The meta-analysis of seven published studies retrieved from the literature search showed a significantly increased RA risk in the recessive model analysis. The IL1-RN VNTR polymorphism was found to be significantly associated with RA susceptibility in Caucasian and Hospital based case-control study (HCC) groups. This meta-analysis showed that IL1-RN VNTR polymorphism may increase the susceptibility to RA**[9-11].**

Our study revealed, genes encoding for IL-1Ra are located on the long arm of chromosome 2 in humans (2q13-14). Polymorphism of the IL-1Ra gene, displays different copies of 86-bp tandem repeat in intron 2. The number of times this sequence is referred to as the copy number of the 86-bp sequence which in turn corresponds to different alleles of this IL-1Ra polymorphism. Five alleles of IL-1RA 86-bp VNTR polymorphism are as follows were classified on basis of number of repeats of 86 bp sequence and PCR product size on gel and designated as A1 (410 bp, 4 repeats), A2 (240 bp, 2 repeats), A3 (500 bp, 5 repeats), A4 (325 bp, 3 repeats) and A5 (595 bp, 6 repeats). All the samples of RA group and HC group were genotyped using PCR for IL-1RA 86-bp VNTR polymorphism using the primers flanking the 86 bp length of DNA of intron 2 of IL-1Ra gene. The PCR products were analyzed on 2% gel along with 100 bp MW marker to confirm the expected product size of each allele present in each sample of patients and control group. In our population we found four alleles (A1, A2, A3 and A4) form seven genotype **[12-14].**

Our statistical data suggested that the Genotype frequencies, allele frequencies and carriage rates of IL-1Ra VNTR alleles are depicted in table no. 4.6. Genotype frequency between rheumatoid arthritis (RA) patient and healthy control groups were slightly different and significantly associated with arthritis ($\chi2$=15.25 P=0.0184*). Thus allele frequency ($\chi2$=15.44 P=0.0015**) and carriage rate ($\chi2$=12.60, P=0.0056**) were also significantly different between both case and control groups. Odds ratio of A1/A1 genotype was indicate protective effects to disease thus A1 (P=0.0033**) allele are also protective allele. The genotype A1/A1 (P=0.0155*) and A1/A4 (P=0.0128*) were significantly associated with arthritis. A1/A1 genotype showed an overall significant difference in both groups with high odds ratio 0.4940 in RA group respectively as compared to controls whereas genotype A2/A2 and A1/A2 were significantly increased in RA compared to HC group. An odds ratio of when compared to RA respectively indicated the protective effect of this genotype. Allele 2 showed a significant increase frequency and carriage rate in disease group as compared to controls. On the contrary allele A1 showed the high frequency and increased carriage rate (75.00%) in control population. Genotype A1/A3 was found no significant differences in distribution of this genotype among control population and RA population (03.12% vs 0.89%) respectively. Genotype A2/A3 was no significantly distributed in HC group as compared to RA group (04.16% vs 0.89 %). Allele A4 (06.25% vs 0.89%) was also found in case and control population**[15].**

IL-1Ra VNTR polymorphism was similar to our study. They revealed polymorphisms in the regulatory regions of cytokine genes are highly influenced by ethnicity. Polymorphisms in interleukin 1-β (IL-1β) and IL-1 receptor antagonist (IL-1Ra) genes, respectively encoding a potent inflammatory agent and an antagonist, which combines with IL-1 receptors competitively, have been associated with a number of diseases like systemic lupus erythematosus, rheumatoid arthritis, sepsis, kidney diseases, and cancer **[17].** In this study, we therefore evaluated the distribution of interleukin-1 gene cluster (IL-1β promoter region, exon-5 and IL-1Ra) gene polymorphisms in 206 healthy north Indian subjects, using PCR-based restriction analysis. They constructed various haplotypes and estimated the linkage disequilibrium (LD). They found genotype and allelic frequencies for these cytokines were conspicuously different when compared among different ethnic populations. Genetic linkage between three loci of IL-1 gene showed strong association among the variants in controls (D′=0.42, *p*<

0.001). Thus they signify an impact of ethnicity and provide a basis for future epidemiological and clinical studies**[16-19].**

Circulating levels of acute phase reactant proteins such as plasma C-reactive protein (CRP) are likely influenced by multiple genes regulating the innate immune response. They screened a set of 16 inflammation-related genes for association with CRP in a large population-based study of healthy young adults (n=1627). In the pooled analysis, the minor allele of *IL1RN* 1018 (rs4251961) within the gene encoding interleukin (IL)-1 receptor antagonist (IL-1RA) was significantly associated with higher mean plasma log (CRP) level ($P < 1x10^{-4}$). The same *IL1RN* 1018 allele was associated with higher mean plasma log (IL-6) levels (P=0.004). In the pooled analysis, the minor allele of *IL1RN* 13888 (rs2232354) was associated with higher fibrinogen, (P=0.001) **[21].**The*IL1RN* 1018 and 13888 variant alleles tag a clade of *IL1RN* haplotypes linked to allele 1 of an 86-bp VNTR polymorphism. The *IL1RN* 1018 variant (rs4251961) was associated with decreased cellular IL-1RA production ex vivo. Common functional polymorphisms of the *IL1RN* gene are associated with several markers of systemic inflammation. Interleukin-1 receptor antagonist (*IL-1Ra*) is a anti-inflammatory cytokine, which inhibits *IL-1* activity by binding to its receptors. The aim of this study was to investigate the association between RPL and *IL-1Ra* intron 2 polymorphism (86 bp VNTR) in Iranian women. In this case control study, genetic polymorphism was studied in 140 RPL patients and 140 healthy women as controls **[22].**Genomic DNA was extracted from the blood samples and polymorphism analysis was performed using the polymerase chain reaction (PCR) method. Finally, the data obtained were analyzed by statistical software. The increased frequency of the IL-1Ra 1/1 genotype in the case group compared to the control group. Whereas, the frequency of IL-1Ra genotype 1/2 was higher in control group than in the case group. However, we did not observe an association between *IL-1Ra* 86 bp VNTR polymorphism in intron 2 and RPL patients (p > 0.05). *IL-1Ra* VNTR polymorphism may not be a genetic factor for RPL**[20-23].**

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