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Genetic Polymorphism of IL-1 β gene and it's association with Rheumatoid arthritis in Vindhyan Population of Madhya Pradesh, India

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ABSTRACT

*Cytokines play a prominent role in etiology and pathogenesis of rheumatoid arthritis (RA), and one of these cytokines is interleukin-1β (IL-1β). The association between IL1B gene single nucleotide polymorphism and rheumatoid arthritis (RA) in a sample of vindhyan patients was investigated. Samples were collected from population of Rewa and Satna district of Madhya Pradesh. Interleukin 1 beta (IL- 1β), a key pro-inflammatory cytokine encoded by the interleukin 1 beta gene, has been associated with chronic inflammation and plays an important role in lung inflammatory diseases including lung cancer. Elevated levels of Interleukin 1proteins, in particular interleukin 1 beta greatly enhance the intensity of the inflammatory response. Biochemical and molecular diagnosis were done using PCR RFLP method. The age, sex, BMI, WHR were the parameters. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of high age group (P<0.0001***), weight of women (P<0.0001***) then men (P=0.5045ns) and BMI of Women (P<0.0001***) then Men (P=0.1913ns). The parameter Waist circumference (cm) and Hip (cm) determine the WHR. Our statistical data from Waist circumference (cm) and Hip (cm) was not significant different between Rheumatoid arthritis (RA) patients group and healthy control (HC) group. Rheumatoid arthritis (RA) patients group 12.50% and also in control group 09.82% (χ^2 = 7.643, P= 0.0219*). 'CT' genotype is higher in patients group then control group (52.08% vs 35.71%) allele frequency was also significantly different (χ^2=5.544, P=0.0185*) whereas carriage rate were not significantly associated (χ^2 = 2.513, P= 0.1129ns). On the basis of our findings we predict association of selected IL-1β gene with pathophysiology of rheumatoid arthritis (RA).*

Introduction

Rheumatoid arthritis (RA) is a worldwide disease with a global prevalence of 0.24% as based on the Global Burden of Disease in 2010, while among Eastern Mediterranean populations; it has been estimated to be 0.37%. It is a chronic inflammatory autoimmune disease characterized by joint swelling, joint tenderness, and destruction of synovial joints, leading to severe disability and premature mortality **[1].**Etiologically, the cause of RA is unknown, but substantial evidence suggests that the disease develops in individuals after interaction between inherited genetic risk factors and environmental triggers. Such interaction can lead to immune dysregulations that are identified as autoantibodies and disturbance of cytokines in serum many years prior to the diagnosis of disease. Accordingly, cytokines have been suggested to play a prominent role in the etiology and pathogenesis of RA, and one of these cytokines is interleukin-1β **[2-4].**

IL-1β belongs to the IL-1 family, which consists of three further interleukins (IL-1α, IL-18, and IL-33). Expression of IL-1α occurs on the surface of the same cell or retains within the cell, while biological actions of IL-1β is produced by acting on other cells. The action of both interleukins can be blocked by an endogenous inhibitor, which is IL-1 receptor antagonist (IL-1Ra). Both types of IL-1 (IL-1α and IL-1β) exert their activity by binding and signaling through two types of cellular receptors; IL-1RI and IL-1RII. The binding of IL-1 to the former receptor results in intracellular signal transduction, while

the latter receptor functions as a decoy receptor for IL-1. In RA patients, IL-1β showed a significant increased level in serum and synovial fluid; and moreover, such increase was positively correlated with the disease severity. A further effect of such cytokine was reported on the capacity of synovial fibroblast to produce cytokines, chemokines, and prostaglandins. In addition, IL-1β can activate osteoclast, and in RA patients, such activation was associated with increased expression of endothelial cell adhesion molecules, and the resulting effects lead to an imbalance in bone metabolism that favors bone resorption and osteoporosis [8]. These observations suggest a role for IL-1 in the pathogenesis of RA **[5-7].**

Genetic studies that were based on single nucleotide polymorphisms (SNPs) of *IL1* genes revealed that genetic polymorphisms of *IL1A, IL1B,* and *IL1RN* genes may have a role in susceptibility to RA. Among these is *ILA* gene polymorphism at positions -899 (C/T) and +4845 (G/T), which showed positive associations with RA (i.e. increased the risk to develop the disease), and moreover, they were associated with altered serum levels of IL-1. Two *IL1B* gene SNPs (-511 C/T and +3953 C/T) were also associated with an increased risk of RA and impacted the disease activity and IL-1β expression. In Vindhyan patients, the *IL1B*-511 *C* allele was reported to have a protective effect against RA development, and in addition, the results suggested a possible role of *IL1B*+3953 CT genotype in the severity of RA [10]. However, these findings were subjected to the effect of the ethnicity of RA patients. Such subject has been inspected by a study that was based on a meta-analysis of 16 published association studies of *IL1A, IL1B* and *IL1RN* gene polymorphisms in RA. For *IL1B*-511 SNP (C/T), a negative association between *T* allele and RA was reported in Caucasian patients, while in Asian RA patients, no such association was observed. However, the TT+TC genotype of *IL1B*-3953 SNP (C/T) showed a positive association between Caucasian and Asian RA patients **[8-12].** Our aim was to investigate the possible association of *IL1B*-511 SNP (C/T) with RA.

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.

Healthy controls

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.

Sample collection

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 (Banglore Genei, 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* III double digest (Banglore Genei, 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 (Vilber Lourmate, 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 control 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 interlukin-1 beta (IL1-β) SNP via PCR-RFLP

The nucleotide position -511 in the promoter region of this gene has a single nucleotide polymorphism that results in change of nucleotide from cytosine (C) to thymine (T). The oligonucleotide sequence (primers) were designed to create a recognition site for the restriction enzyme *Ava*I in allele 1 (C at -511 position) but no restriction site in allele 2 (T at -511 position) of IL-1β gene.

Primer sequences

The oligonucleotides sequences (primers) used were those described by (Adeel Gulzar Chaudhary, 2008):

IL1- β forward primer - 5' TGGCATTGATCTGGTTCATC 3'
IL1- β reverse primer - 5' GTTTAGGAATCTTCCCACTT 3'

PCR Mix

For each DNA sample 25 µl of PCR reaction mixture was prepared containing 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 (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for IL1- β gene, 0.2 µl of 5U/ µl of *Taq* DNA polymerase (final concentration 1U; Genetix Biotech Asia Pvt. Ltd.,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 2 min and 35 cycles of denaturation at 95˚C for 1 min, annealing at 54˚C for 1 min and extention at 74˚C for 1 min, followed by final extension at 74˚C for 10 min. PCR products were separated on 2% agarose gel (2% w/v, Sigma) using a 100 bp molecular weight (MW) marker to confirm the PCR product size of 304 bp.

Restriction Digestion by AvaI

The C to T transition in promoter region of IL1- β gene when amplified by PCR was than incubated with *Ava*I restriction enzyme (New England Biolabs,USA). The reaction mix included 0.3 µl of 10,000U/ml *Ava*I restriction enzyme (final concentration 3U), 2.0 µl of 10X NEBuffer 4 (final concentration 1X; 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9), 12.0 µl of PCR product and 5.7 µl of sterile water. Reaction was incubated for 16 hrs at 37˚C for complete digestion. 10 µl of digested PCR product was loaded on 9% polyacrylamide gel. Electrophoresis was done at 80 V in 1X Tris-borate EDTA buffer (89 mM Tris pH 7.6, 89 mM Boric acid, 2 mm EDTA pH 8.0). A 100 bp gene DNA ladder (Roche, Germany) was run concurrently as molecular weight marker. The gel was than stained with ethidium bromide (10mg/ml). The products were visualized using an ultraviolet trans illuminator. The gel picture was captured using a digital camera and gel documentation software (Vilber Lourmate, Cedex I, France).

Genotyping

Digestion of the amplified 304 bp PCR product gave two fragments of 190 bp and 114 bp respectively if the product was excisable by *Ava* I. Depending on digestion pattern, individuals were scored as genotype CC when homozygous for presence of *Ava* I site, genotype TT when homozygous for absence of *Ava* I site and genotype CT in case of heterozygosity.

Results

This study based on comparative statistical analysis between Rheumatoid arthritis (RA) patients group and healthy control (HC) group. Comparative Statistical analysis of anthropometric and clinical parameter may give information about association with Rheumatoid arthritis. Thus genetic association of some genes such IL-1β, IL-1Ra, PTPN22 and STAT4 would reveal significant differences between the groups. This population based study revealed genetic association in Vindhyan Population.

Anthropometric results

The descriptive data and comparison of anthropometric and biochemical parameters of Rheumatoid arthritis (RA) patients versus controls are presented in Table no.1. The age, sex, BMI, WHR were the parameters. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of high age group (P<0.0001***), weight of women (P<0.0001***) then men (P=0.5045ns) and BMI of Women (P<0.0001***) then Men (P=0.1913ns). The parameter Waist circumference (cm) and Hip (cm) determine the WHR. Our statistical data from Waist circumference (cm) and Hip (cm) was not significant different between Rheumatoid arthritis (RA) patients group and healthy control (HC) group. WHR was not significantly different between patient and healthy population. Thus WHR in Women (P=0.2904ns) and Men (P=0.1912ns) were not found were not found association with Rheumatoid arthritis (RA) **(See Table No. 1).**

Table 1. Comparison studies of anthropometric parameters between Rheumatoid arthritis (RA) patients and healthy controls.

n(Men/Women)	96(54/42)	112(62/50)	
Age(years)	59.6±12.4	52.5±12.5	P<0.0001***
Height(m)	162.50±11.3	161.2±12.4	0.4333,ns
Weight (Kg)			
Women	69.5 ±4.7	60.6 ± 4.5	P<0.0001***
Men	68.4±5.6	67.8±7.1	0.5045,ns
BMI (kg/m^2)			
Women	25.6±3.1	22.1 ± 4.3	P<0.0001***
Men	24.6±4.7	23.8± 4.1	0.1913,ns

Waist circumference (cm)			
Women	92.5±6.2	93.6±6.7	0.2233,ns
Men	90.0±7.0	89.0±6.0	0.2685,ns
Hip (cm)			
Women	95.9±2.4	96.1±2.2	0.5315,ns
Men	90.8±4.3	91.2±1.5	0.3579,ns
WHR			
Women	0.97±0.05	0.98±0.08	0.2904,ns
Men	0.98±0.08	0.99±0.01	0.1912,ns

*(*denotes level of significant change between case and control)*

Biochemical and clinical findings

Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of Rheumatoid arthritis (RA) patients versus healthy controls are presented in Table no. 2. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of ESR (P<0.0001***), α $_1$ACT (P<0.0001***) and Serum CRP (P<0.0001***) compared to that of control subject. Thus P-Value of ESR (Erythrocyte sedimentation rate) , α $_1$ACT (Alpha 1 antichymotrypsin) and CRP (C-Reactive Protein) concentration revealed association significantly with Rheumatoid arthritis (RA) patients. Whereas rest of all parameters such as Post-Prandial Glucose (mg/Dl), HbA1C(%), HDL-C(mmol/L), LDL-C (mg/dL), TG(mg/dL), Systolic BP (mmHg), Diastolic BP (mmHg) and Blood Urea(mg/dL) were not significantly different between patient and healthy population **(See Table No. 2).**

Table 2. Comparison of Biochemical and clinical findings of Rheumatoid arthritis (RA) patients and Healthy controls.

Post-Prandial Glucose (mg/Dl)	117.7±12.4	118.4±11.6	0.6747,ns
HbA1C(%)	5.8±0.7	5.7±0.8	0.3424,ns
HDL-C(mmol/L)	108.8±12.2	109.3±11.6	0.7625,ns
LDL-C (mg/dL)	42.1±2.6	41.8±3.7	0.5063,ns
TG(mg/dL)	125.9±13.2	126.2±12.2	0.8650,ns
Systolic BP (mmHg)	125.4±8.1	124.8±5.7	0.5332,ns
Diastolic BP (mmHg)	87.1±5.8	86.5±6.2	0.4744,ns
Blood Urea(mg/dL)	18.1±1.7	17.8±1.8	0.2204,ns
ESR, mm/h	63.58±15.34	45.62±18.23	P<0.0001***
α $_1$ACT (mg/dL)	1.9±0.41	1.2±0.35	P<0.0001***
Serum CRP, (mg/dL)	14.5±3.7	12.7±2.6	P<0.0001***

(denotes the level of significant change between case and control)*

Detection of Genetic Polymorphism in IL-1 β Gene

PCR amplification of IL-1 β Gene with specific primers gave 304-bp product which was digested with *AvaI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 109 and 104 bp. The heterozygous genotype (TC) was represented as 3 fragments of 304, 190 and 104 bp of DNA fragments in the gel **(Depicted in figure no. 1.)**

The distribution of the polymorphisms of IL-1 β C511T was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for IL-1 β polymorphism are depicted in table 3 and table 4and Graph 4.1, 4.2, 4.3. Significant level of change has been seen in overall distribution of IL-1 β genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'CC' genotype as compared to Patients of Rheumatoid arthritis (54.46% *vs* 35.41%). Similarly, mutant type 'TT' genotype was present in low frequency in Rheumatoid arthritis (RA) patients group 12.50% and also in control group 09.82% (χ^2 = 7.643, P= 0.0219*). 'CT' genotype is higher in patients group then control group (52.08% *vs* 35.71%) allele frequency was also significantly different (χ^2=5.544, P=0.0185*) whereas carriage rate were not significantly associated (χ^2 = 2.513, P= 0.1129ns).

An odds ratio of CC genotype was calculated as 0.4585 which indicates little protective effect of CC genotype from disease and indicate significantly differences between patient and healthy group (P=0.0079**). An odds ratio of TC and TT genotype was not indicate as protective effect. Significant difference in Allele frequency between Rheumatoid arthritis (RA) patients group and healthy control group indicate association with Rheumatoid arthritis (P=0.0211*) but not show protective effect whereas carriage rate was not significantly different. Overall allele 'C' was found little lower frequency in disease group as compared to HC group (61.45% Vs 72.32%) whereas allele '*T'* was present in little high frequency in the disease group was significantly different. Carriage rate of allele 'T' was slightly high in Rheumatoid arthritis (RA) group as compared to healthy control (42.46% Vs 33.55%) whereas carriage rate of allele '*C*' was nominal different in both control

and disease group but no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests IL-1 β polymorphism is significantly associated with Rheumatoid arthritis in our population **(See Table No. 3 and 4).**

Table 3. Frequency distribution and association of Genotype, allele frequency and carriage rate of IL-1 β gene polymorphism in population of Vindhyan region using Chi Square Test

IL-1 β GENE	CASE N= 96		CONTROL N=112		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
CC	34	35.41	61	54.46	
CT	50	52.08	40	35.71	
TT	12	12.50	11	09.82	7.643(0.0219*)
Allele					
C	118	61.45	162	72.32	
T	74	38.54	62	27.67	5.544 (0.0185*)
Carriage Rate					
C	84	57.53	101	66.44	
T	62	42.46	51	33.55	2.513 (0.1129ns)

(- 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 4. Fisher Exact Test values of IL-1 β gene polymorphism

IL-1 β GENE	CASE N= 96		CONTROL N=112		P Value	Odds Ratio (95% confidence interval)
	N	%	N	%		
Genotype						
CC	34	35.41	61	54.46	0.0079**	0.4585 (0.2619 to 0.8025)
CT	50	52.08	40	35.71	0.0245*	1.957 (1.121 to 3.414)
TT	12	12.50	11	09.82	0.6584ns	1.312 (0.5506 to 3.125)
Allele						
C	118	61.45	162	72.32		0.6103 (0.4040 to 0.9220)
T	74	38.54	62	27.67	0.0211*	1.639 (1.085 to 2.475)
Carriage Rate						
C	84	57.53	101	66.44		0.6841 (0.4275 to 1.095)
T	62	42.46	51	33.55	0.1218ns	1.462 (0.9133 to 2.339)

(- 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.)

Discussion

Rheumatoid arthritis (RA) is a chronic auto-inflammatory disease affecting connective tissue, characterized by progressive joint damage and specific systemic disorders. To date, the number of patients with autoimmune arthritis is almost 1% of the world's population, establishing an urgent problem for healthcare systems worldwide **[15].** An evident feature of RA is clinical polymorphism, represented by a wide variability of symptoms, clinical forms, and progression rates. RA is considered to be a multi-factorial disease triggered by a genetic predisposition and environmental factors. Both clinical and genealogical studies have shown that the disease can accumulate in families. This relationship has been confirmed by modern molecular genetics. The opportunity to identify RA risk groups in different populations, as well as the possible prognostic value of some genetic variants for disease development, progression, and treatment, including a personalized anti-rheumatic therapy response, has promoted new studies of germ line genetic variants in RA patients. Rheumatoid arthritis (RA) is the most common inflammatory arthropathy worldwide **[13-16].** A significant number of RA-associated genetic variants have been identified in the interleukins (ILs) and their receptor genes. Interleukins are cytokines that stimulate hematopoietic cell development and T- and B-lymphocyte differentiation. There are seven interleukin families characterized by significant

variability in both ligand forms and receptors. The key mediator of inflammation, IL1, is represented by two isoforms and is combined with another nine interleukins in one family. Its expression is affected by the –511A/G (rs16944) polymorphism in the promoter (associated with RA in Caucasians) and the +3953C/T polymorphism in exon 5 (RA-associated in some Asian populations). According to meta-analyses, RA-associated alleles in Caucasians include –174G/C and –572G/C of the IL6 gene, rs1800896 in the IL10 gene, rs13151961 and rs6822844 located near the IL2 and IL21 genes, rs7530511 and rs11209026 in the IL23R gene, and some others. A significant number of RA-associated polymorphic loci included in the meta-analyses are unique to Asian populations. The polymorphism found at rs1946518 of the IL18 gene is found in Egyptian populations, and the polymorphism found at rs549908 is found in the Taiwanese population. Thus the candidate genes for assessing genetic RA predisposition need to be thoroughly selected **[17-19].**

Our data from clinical parameters revealed Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of Rheumatoid arthritis (RA) patients versus healthy controls are presented in Table no. 4.2. As expected the Rheumatoid arthritis (RA) patients had markedly higher levels of ESR (P<0.0001***), α $_1$ACT (P<0.0001***) and Serum CRP (P<0.0001***) compared to that of control subject. Thus P-Value of ESR (Erythrocyte sedimentation rate) , α $_1$ACT (Alpha 1 antichymotrypsin) and CRP (C-Reactive Protein) concentration revealed association significantly with Rheumatoid arthritis (RA) patients. Whereas rest of all parameters such as Post-Prandial Glucose (mg/Dl), HbA1C(%), HDL-C(mmol/L), LDL-C (mg/dL), TG(mg/dL), Systolic BP (mmHg), Diastolic BP (mmHg) and Blood Urea(mg/dL) were not significantly different between patient and healthy population. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) can be considered as inflammation indicators in RA patients. In addition, most of adipokines are involved in modulation of inflammation and thus might hypothetically play a role in RA pathogenesis8. Some studies have shown that adipokines including leptin, resistin, and visfatin may contribute to RA pathophysiology. Obesity and increased body fat mass are associated with higher production of adipokines with pro-inflammatory characteristics. In this study, two other controversial adipokines, namely nesfatin-1 and asymmetric dimethylarginine (ADMA), were evaluated to reveal the possible relationships between them and RA activity **[25].**

Rheumatoid arthritis (RA) is a chronic, systemic, and autoimmune disease that affects around one percent of adults worldwide and is associated with disability, loss of productivity, and decreased life expectancy and quality. The etiology of RA is not clear, while the development of disease may be affected by both genetic susceptibility and life style risk factors including nutrition. There has been a long-standing debate whether diet plays a pivotal role in autoimmune diseases including RA **[18,20].**Various dietary patterns and some specific nutrients are speculated to be relevant to the occurrence of the disease. Although none of these diets or nutrients has been proven to be clinically effective so far, there is no doubt all RA patients can benefit from a balanced diet. Our statistical data from comparison of anthropometric and biochemical parameters of Rheumatoid arthritis (RA) patients versus controls was suggested that the number Rheumatoid arthritis (RA) patients had markedly higher in high age group (P<0.0001***), weight of women (P<0.0001***) was high then men (P=0.5045ns) and BMI (Body mass index) of Women (P<0.0001***) was also high then Men (P=0.1913ns). The parameter Waist circumference (cm) and Hip (cm) determine the WHR. Our statistical data from Waist circumference (cm) and Hip (cm) was not significant different between Rheumatoid arthritis (RA) patients group and healthy control (HC) group. Thus WHR in Women (P=0.2904ns) and Men (P=0.1912ns) were not found association with Rheumatoid arthritis **[21-24].**

Our result from IL-1 β Gene polymorphism revealed genetic association with Rheumatoid arthritis (RA). The PCR amplification of IL-1 β Gene with specific primers gave 304-bp product which was digested with *AvaI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 109 and 104 bp. The heterozygous genotype (TC) was represented as 3 fragments of 304, 190 and 104 bp of DNA fragments in the gel. The distribution of the polymorphisms of IL-1 β C511T was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for IL-1 β polymorphism are depicted in table 4.4 and table 4.5 and Graph 4.1, 4.2, 4.3. Significant level of change has been seen in overall distribution of IL-1 β genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'CC' genotype as compared to Patients of Rheumatoid arthritis (54.46% *vs* 35.41%). Similarly, mutant type 'TT' genotype was present in low frequency in Rheumatoid arthritis (RA) patients group 12.50% and also in control group 09.82% (χ^2 = 7.643, P= 0.0219*). 'CT' genotype is higher in patients group then control group (52.08% *vs* 35.71%) allele frequency was also significantly different (χ^2=5.544, P=0.0185*) whereas carriage rate were not significantly associated (χ^2 = 2.513, P= 0.1129ns).

Data from fisher exact test suggested that odds ratio of CC genotype was calculated as 0.4585 which indicates little protective effect of CC genotype from disease and indicate significantly differences between patient and healthy group (P=0.0079**). An odds ratio of TC and TT genotype was not indicating as protective effect. Significant difference in Allele frequency between Rheumatoid arthritis patients group and healthy control group indicate association with Rheumatoid arthritis (P=0.0211*) but not show protective effect whereas carriage rate was not significantly different. Overall allele 'C' was found little lower frequency in disease group as compared to HC group (61.45% Vs 72.32%) whereas allele '*T'* was present in little

high frequency in the disease group was significantly different. Carriage rate of allele 'T' was slightly high in Rheumatoid arthritis group as compared to healthy control (42.46% Vs 33.55%) whereas carriage rate of allele '*C*' was nominal different in both control and disease group but no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests IL-1 β polymorphism is significantly associated with Rheumatoid arthritis in our population.

The Cytokines play a prominent role in etiology and pathogenesis of rheumatoid arthritis (RA), and one of these cytokines is interleukin-1β (IL-1β). The association between *IL1B* gene single nucleotide polymorphism (SNP: rs16944) and rheumatoid arthritis (RA) in a sample of Iraqi patients was investigated. Fifty-one RA patients (21 males and 30 females) were enrolled and their age range was 20 - 63 years (44.9 ± 1.5 years) In addition to patients, 45 apparently healthy control subjects were also enrolled in the study **[27].** They matched patients for ethnicity (Iraqis), gender (14 males and 31 females) and age (41.3 ± 1.3 years). Analysis of Hardy-Weinberg equilibrium (HWE) in RA patients and controls revealed that the *IL1B* genotypes were consistent with the equilibrium, and no significant differences ($p > 0.05$) were observed between the observed and expected genotype frequencies. Inspecting *IL1B* genotype and allele frequencies in RA patients and controls revealed that there were no significant variations between these frequencies, although a decreased frequency of *T* allele (67.7 *vs*. 73.3%) and an increased frequency of *C* allele (32.3*vs*. 26.7%) were observed in patients compared to controls **[26-28**].

Conclusion

Although the sample size was lower but genotype distribution was significantly different showing association of IL1B gene with RA.

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