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Interleukin-4 (IL-4) and Interleukin-4 Receptor Alpha Chain (IL-4Rα) Gene Polymorphisms in Egyptian Rheumatic Arthritis Patients

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ABSTRACT

Rheumatoid arthritis (RA) is considered a Th1-driven disease. Interleukin 4 (IL-4) binds to its receptor, promoting Th2 differentiation and limiting Th1 responses, but its role in the pathogenesis of RA is conflicting. *Objective:* To evaluate the occurrence of interleukin-4 (IL-4) and interleukin-4 receptor alpha chain (IL-4Rα) gene polymorphisms in patients with RA and their possible contribution to disease severity. *Methods:* We analyzed 2 polymorphisms in each of the IL4 and IL-4Rα genes in patients with RA and in a control population, as well as measuring serum rheumatoid factor (RF) as a disease severity parameter. *Results*: The IL-4 –590 TT genotype (P<0.001) and the IL4 –590T allele (OR 2.84, 95% CI 1.0-8.77, p = 0.03) were significantly more frequent in patients with RA than in controls, this is similar for IL-4 VNTR RP1⁄RP1 genotype (P<0.001) and the IL-4 RP1 allele (OR 2.91 CI 0.92-10.23, p = 0.04). Higher frequency of IL-4Rα +148 A/G genotypes (p = 0.02) in RA patients compared with controls was also found. Nevertheless, the more severe form of RA was observed in patients carrying the IL-4 -590 T allele as compared with homozygous patients. The IL-4Rα +1902 A allele and IL-4Rα +148G allele were significantly associated with the severe form of RA. *Conclusion*: The IL-4 -590 C/T, IL-4 VNTR in intron-3 and IL-4Rα +148 A/G polymorphisms were associated with RA susceptibility in Egyptian population. IL-4Rα +148 A/G and IL-4Rα +1902 A/G polymorphisms may be genetic risk factors for RA severity.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory polyarthritis that affects 0.5–1% of the general population with high personal, social and economic costs. It is a multifactorial disease, with genetic and environmental factors contributing to the pathogenesis [1].

It is considered that RA is orchestrated by Th1 CD4+ cells that stimulate monocytes, macrophages, synovial fibroblasts, and other cells to produce cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-15, IL-17, and metalloproteinases that produce tissue damage [2]. In response to this proinflammatory reaction, anti-inflammatory mechanisms are activated in an attempt to control the autoreactivity. These mechanisms include the production of IL-10, IL-13, IL-4, and transforming growth factor-ß, as well as antagonists of the mediators of inflammation such as the IL-1 receptor antagonist (IL-1Ra) and soluble receptors of TNF-α [3].

Interleukin-4 (IL-4) is produced by Th2 cells, basophils, eosinophils and mastocytes and promotes the change of immunoglobulin production of B cells from IgM to IgE and IgG4 [4]. It has stimulatory and inhibitory effects, inducing differentiation of Th0 to Th2 cells and among its anti-inflammatory processes are down regulation of proinflammatory cytokines such as TNF-α, IL-1ß, IL-6, and interferon-γ (IFN-γ), inhibition of the proliferation of fibroblastic cells of the articular synovium, and diminution of bone resorption [5]. It has been established that synovial concentrations of IL-4 are low or absent in patients with RA [6].

IL-4 exerts its biological activity by binding to the target cell receptor IL-4R [7]. IL-4R is composed of two subunits: an α subunit that bind IL-4 and transduces its growth promoting and transcription activating functions [8] and a γ subunit that is common to several cytokine receptors and amplifies signaling of the α subunit [9].

The gene for IL-4 has been mapped to the q arm (q23-31) of chromosome 5. A functional polymorphism

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representing C to T substitution at position -590 has been described in the promoter region of IL-4 [10].

One important polymorphism is located in the intron 3 of IL-4 gene and is composed of a 70-bp sequence of variable number of tandem repeats (VNTR) (GT repeat) [11]. It has been proved that the IL-4 intron 3 polymorphism might influence the production of IL-4, with the RP1 (two 70-bp repeats) (183 bp) allele enhancing IL-4 expression compared with RP2 (three 70-bp repeats) (253 bp) allele [11].

The IL-4-590 promoter polymorphism has been suggested to be associated with increased risk of RA and therefore used as genetic marker for assessing the susceptibility and severity of RA in Chinese [12].

IL-4Rα is located at the short arm of chromosome 16p12.1. Several polymorphisms have been described in the coding and in the non-coding regions of the gene. It has been established that polymorphisms located in the +148 coding region (transition A/G, I50V) of the extracellular domain and in the +1902 codifying region {transition A/G, Gln576Arg (Q576R)} of the intracellular domain affect the binding of its ligand or the intracellular signaling, respectively [13].

In this study, we tested the allelic distribution of these four polymorphisms among RA patients and investigated their involvement with RA severity.

Subjects and methods

Patients and controls

The study included 200 patients with RA (75.56% women) with a mean age of (47.75 ± 9) years and 200 healthy controls (73% women) with a mean age of (46.68 ± 6) years. The diagnosis of RA was conformed to the American college of rheumatology criteria [14].

Patients were recruited from the outpatient and inpatient populations of Rheumatology and Rehabilitation Department, Zagazig university hospital, El-Sharqia, Egypt. All the patients and healthy individuals gave their written consent before blood sample collection.

Subjects enrolled in the study underwent routine biochemical blood analysis. X-ray images of wrists, hands and feet were obtained for all patients. The evaluation of subjects included physical examination with particular focus on the pattern of joint involvement, the presence of nodules and other extra-articular features.

Disease severity parameters were determined on the basis of defined parameters and a global physician's assessment. Rheumatoid factor (RF) titers were measured from serum samples by latex agglutination (Humantex RF, Germany). Titers ≥ 20 IU/ml were considered positive, the mean values were used.

Genotyping:

Subjects were genotyped for IL-4 promoter single nucleotide polymorphism at position -590 [15] by PCR-RFLP. The primer pairs employed were forward (A) and reverse (B) (Bio Basic Inc., Ontario, Canada). The primer sequences used for the IL-4 promoter SNP C/T at position -590 were as follows:

5'-ACTAGGCCTCACCTGATACG-3' (forward primer) and 5'-GTTGTAATGCAGTCCTCCTG-3' (reverse primer).

PCR was performed in a final volume of 25 µL containing 7 µL of sterile deionized water, 5 µL of genomic DNA (50 to 100 ng) , 0.5 µL (0.5 µg/µl) of each primer and 12.5 µL of 2X PCR Master Mix (Bioron, Ludwigshafen am Rhein, Germany). The amplification was carried out using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the following protocol: denaturation at 95˚C for 0.5 minute, annealing at 57˚C for 0.5 minutes and extension at 77˚C for 0.5 minutes with 32 cycles. Digestion was performed with 1 µL *Bsm*F1 restriction enzyme (fermentas, Germany) in a total volume of 20 µL that contain 10 µL of PCR product and 2 µL of 10X Fast Digest Green buffer and 7 µL of nuclease free water. The components were mixed gently and incubated at 37ºC in a heat block for 5 min. Digestion yielded 252 bp for the T allele and 192 + 60 bp fragments for the C allele. The products were then stored at 4ºC until use. The digested products were analyzed on 3% agarose gels stained with ethidium bromide using, a 50 bp Marker.

Subjects were also genotyped for IL-4 VNTR (70 bp repeat in intron 3) with the RP1 (two 70-bp repeats) allele and RP2 (three 70-bp repeats) allele by PCR [10]. The primer pairs employed were forward primer: 5'-AGGCTGAAAGCGGGAAAGC-3' and reverse primer: 5'-CTGTTCACCTCAACTGCTCC-3' (Bio Basic Inc., Ontario, Canada). PCR protocol: denaturation at 95˚C for 0.5 minute, annealing at 60˚C for 0.7 minutes and extension at 72˚C for 0.7 minute with 30 cycles. PCR products yield 183 bp fragment for RP1 allele (two 70-bp repeats) and 253 bp fragment correspond to RP2 allele (three 70-bp repeats). PCR products were directly analyzed on 2% agarose gels stained with ethidium bromide, using a 100 bp Marker, and each allele was recognized according to its size.

Subjects were genotyped for IL-4Rα gene +148 A/G single nucleotide polymorphism of the +148 coding region +148 A/G by PCR-RFLP [16]. The region surrounding the polymorphism was amplified with the following forward primers 5'-GGCAGGTGTGAGGAGCATCC-3' and reverse primer 5'-GCCTCCGTTGTTCTCAGGGA-3' (Bio Basic Inc., Ontario, Canada). PCR was performed in a final volume of 25 µL containing 7 µL of sterile deionized water, 5 µL of genomic DNA, 0.5 µL (0.5 µg/µl) of each primer, and a 12.5 µL of 2X PCR Master Mix (Bioron, Ludwigshafen am Rhein, Germany). The amplification was carried out using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) at 93˚C for 5 min, followed by 36 cycles at 93˚C for 60 sec, at 60˚C for 60 sec and 72˚C for 60 sec. Digestion of 273 bp-amplified product with *Rsa*I (fermentas, Germany) yielded 273 bp for the A allele and 254-bp fragment when the G allele was present. The PCR products were separated on 2% agarose gels stained with ethidium bromide using a 100 bp Marker.

Subjects were genotyped for IL-4Rα gene single nucleotide polymorphism at position 1902 (codon 576) A/G transition (Q576R) by PCR-RFLP [17] using forward primers 5'-GCCCCCACCAGTGGCTACC-3' and reverse primers 5'-GCCTTGTAACCAGCCTCTCCT-3' (Bio Basic Inc., Ontario, Canada). PCR conditions were as follows, denaturation at 94˚C for 0.5 min, annealing at 55˚C for 0.5 min and extension at 72˚C for 0.5 min for 30 cycles. Digestion of the 123 bp-amplified product with *Msp*I (fermentas, Germany) yielded 107 + 16 bp for Q (A) allele and 89 +18 +16 bp fragments for R (G) allele. The products were then stored at 4ºC until use. The PCR products were separated on 3% agarose gels and visualized under ultraviolet illumination after staining with 0.4 mg/L ethidium bromide. The agarose concentration and DNA molecular weight markers for each genotyping were adapted to the size of the expected DNA products.

Statistical analysis:

All results were analyzed by SPSS software (version 14) [18]. Results of the gene polymorphism studies were analyzed by comparison of allele frequencies (number of copies of a specific allele divided by the total number of alleles in the group) and carriage rates (number of individuals with at least 1 copy of a specific allele divided by the total number of individuals within the group) were calculated in the RA and control groups. Chi-square test was used for comparisons of frequencies between RA patients and healthy controls. Odds ratios (OR) were calculated for disease susceptibility in carriers of specific alleles. The 95% confidence intervals (CI) for the OR were also calculated. A P-value <0.05 was considered to be statistically significant. RF titers as a disease severity parameter were expressed as mean ± SD. Comparison of mean values of studied variables among different groups was done using ANOVA test.

Results

Agarose gel electrophoresis detection for IL-4 promoter single nucleotide polymorphism at position -590 for rheumatoid arthritis patients showed that lanes of 252 bp fragment for TT homozygous genotype on the other hand, 252 + 192 + 60 bp fragments for CT heterozygous genotype and lanes of 192 + 60 for CC genotype (50 bp Marker), moreover, lanes 2, 3, 4, 5, 7, 8, 9, 10, 11, 14, 15, 19 and 20 homozygous for TT (252 bp, lane 16 homozygous for CC and lanes 1, 6, 12, 13, 17, 18 and 21 heterozygous CT (**Figure 1**).

Figure 2 shows IL-4 VNTR (intron 3) for rheumatoid arthritis patients and lanes of 183 bp fragment for RP1/RP1 homozygous genotype, 253 bp fragment for RP2/RP2 genotype and lanes of 183 + 253 for RP1/RP2 heterozygous genotype. Tested samples in lanes 1, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 17, 18, 19 and 21 were homozygous for RP1/ RP1, lanes 2, 8, 10, 15, 16 and 20 heterozygous for RP1/RP2 and RP2/RP2 homozygous signals not detected.

Figure 3 shows polymorphism of IL-4Rα at position +1902 where lanes of 107 bp fragment for AA homozygous genotype, 89 bp fragment for GG genotype and lanes of 107 + 89 for AG heterozygous genotype.

From **Fig. 3** we showed that, lanes 1, 2, 3, 4, 5, 7, 8, 10, 12, 13, 14, 15, 16, 17, 18, 20 and 21 homozygous for GG genotype and lanes 6, 9, 11 and 19 heterozygous for AG genotype, but AA homozygous was undetected. On the other hand, agarose gel electrophoresis detection for IL-4Rα polymorphism at +148 showed lanes 2, 4, 6 and 18 homozygous for AA, lane 9 homozygous for GG and lanes 1, 3, 5, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20 and 21 heterozygous for AG.

Agarose gel electrophoresis detection for IL-4Rα polymorphism at position +148 for rheumatoid arthritis patients (**Fig. 4)** showed lanes of 254 bp fragment for GG homozygous genotype, 273 bp fragment for AA genotype and lanes of 254 + 273 for AG heterozygous genotype, the result showed that, lanes 2, 4, 6 and 18 homozygous for AA, lane 9 homozygous for GG and lanes 1, 3, 5, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20 and 21 heterozygous for AG.

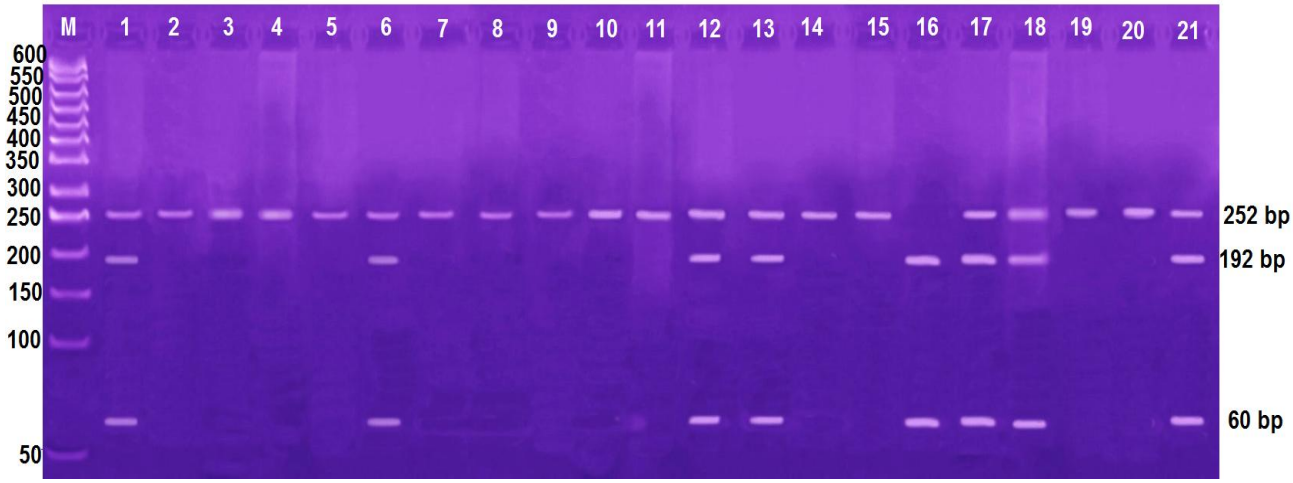

Fig. 1: Agarose gel electrophoresis detection for IL-4 promoter single nucleotide polymorphism at position -590 for rheumatoid arthritis patients showing lanes of 252 bp fragment for TT homozygous genotype, 252 + 192 + 60 bp fragments for CT heterozygous genotype and lanes of 192 + 60 for CC genotype (50 bp Marker)

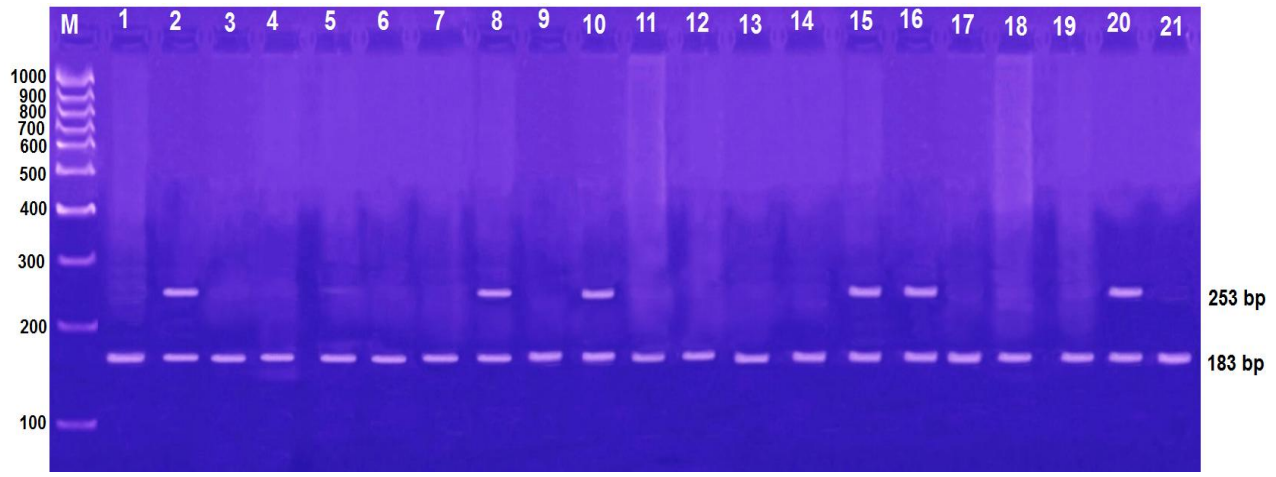

Fig. 2: Agarose gel electrophoresis detection for IL-4 VNTR (intron 3) for rheumatoid arthritis patients showing lanes of 183 bp fragment for RP1/RP1 homozygous genotype, 253 bp fragment for RP2/RP2 genotype and lanes of 183 + 253 for RP1/RP2 heterozygous genotype (100 bp Marker).

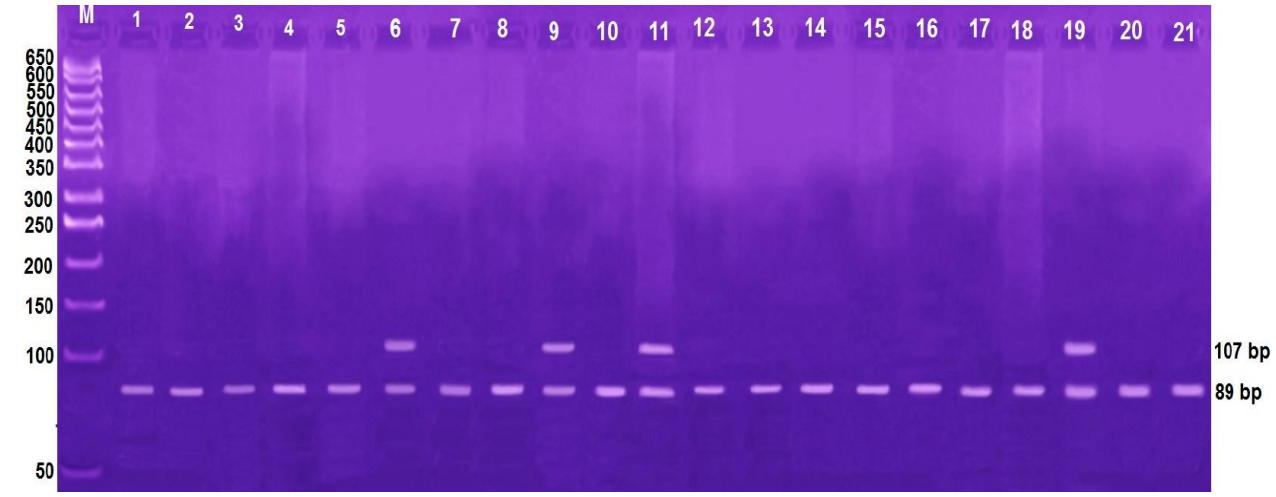

Fig. 3: Agarose gel electrophoresis detection for IL-4Rα polymorphism at position +1902 for rheumatoid arthritis patients showing lanes of 107 bp fragment for AA homozygous genotype, 89 bp fragment for GG genotype and lanes of 107 + 89 for AG heterozygous genotype (50bp Marker).

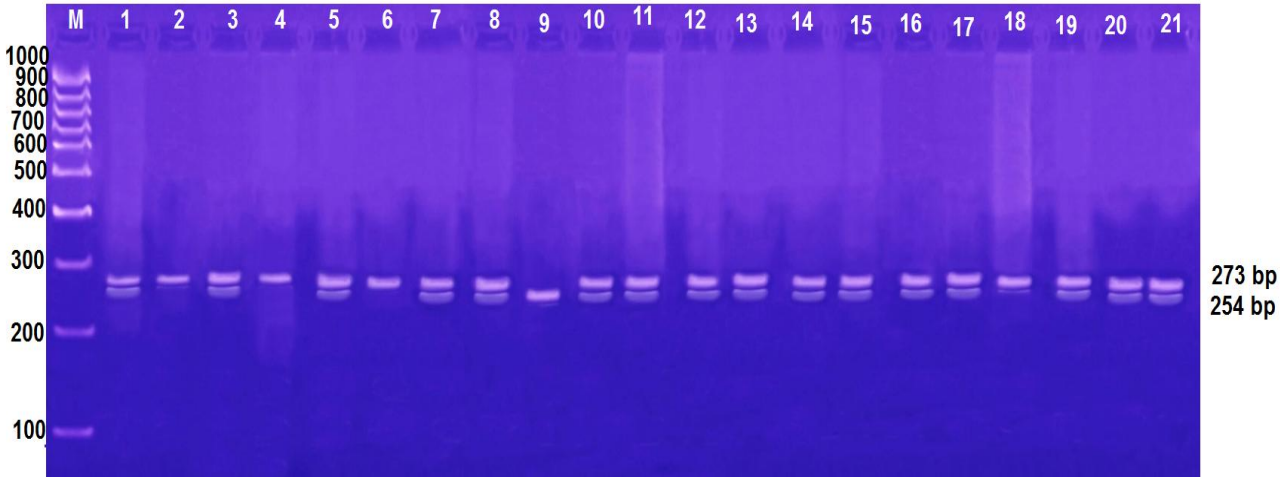

Fig. 4: Agarose gel electrophoresis detection for IL-4Rα polymorphism at position +148 for rheumatoid arthritis patients showing lanes of 254 bp fragment for GG homozygous genotype, 273 bp fragment for AA genotype and lanes of 254 + 273 for AG heterozygous genotype (100 bp Marker).

RP1/RP1 genotype for IL-4 VNTR was found to be more frequent (56.5%) than both RP1/RP2 and RP2/RP2 genotypes (36.5% and 7%, respectively) among RA patients. Moreover, IL-4 promoter single nucleotide polymorphism at position -590 showed significant difference in frequency of TT genotype (53.33%) that was higher than CT and CC (35.56% and 11.11%, respectively). IL-4Rα +1902 observed that, GG genotype (49%) is more frequently than AA and AG. While IL-4Rα (at position +148) showed AG (53%) genotype more frequent among RA patients than GG and AA genotypes (**Table 1**).

The frequency of IL-4 RP1 allele is significantly higher than RP2 allele (P=0.04) otherwise, the frequency of IL-4 -590 T allele is higher than IL-4 -590 C allele (p = 0.03) among the studied RA cases and IL-4 VNTR RP1/RP1 genotype (p <0.001) and IL-4 RP1 allele (OR 2.91 CI 0.92-10.23, P = 0.04) were significantly more frequent in patients with RA than in controls. The IL-4 –590 TT genotype (p <0.001) and the IL4 –590T allele (OR 2.84, 95% CI 1.0-8.77, p = 0.03) were significantly more frequent in patients with RA than in controls (**Table 2**).

On the other hand, IL-4 -590 T allele (TT), IL-4Rα +1902A (AA) allele and IL-4Rα +148 G (GG) allele carriers showed the higher RF titers (**Table 3**).

The simultaneous carriage of these two alleles was observed in 167 patients (83.5%) and 11 patients (5.5%) carrying IL-4 RP1 allele, 20 patients (10%) were carrying IL-4 -590 T allele compared with only two patients carrying none of the two alleles indicating a relationship between the two alleles in RA patients (**Table 4**). It was observed that most RA patients (83.33%) are carrying both IL-4 RP1 and IL-4 -590 T alleles where only one patient was not carrying any of these two alleles (**Figure 5**).

Discussion

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and pannus formation, which can lead to severe destruction of cartilages and bones [19].

The imbalance between pro-inflammatory and anti-inflammatory cytokines is a feature of RA .The role of IL-4 and its receptor in the pathogenesis of RA is conflicting. The aim of this study was to evaluate the occurrence of IL-4 and IL-4Rα variants in RA patients and their possible contribution to RA susceptibility and/or severity.

A single nucleotide polymorphism (SNP) at position -590 of the promoter region, a 70-bp variable number of tandem repeat (VNTR) in intron 3 for IL-4 and another two SNPs of A to G at position +1902 and A to G at position +148 for IL-4RA were also evaluated.

Our results revealed a significantly higher frequency of the IL4 –590TT genotype (P <0.001), a significantly higher frequency of the IL-4–590T allele in RA patients compared with controls.

Other groups have also suggested association of IL-4–590T allele with RA and palindromic rheumatism, and with greater disease activity [20-22]. In addition, Nuñez et al., [23] reported that IL-4 −590 C/T SNP was associated with type1 diabetes and rheumatoid arthritis.

Table 1: Distribution of genotypes among the rheumatoid arthritis (RA) and healthy control subjects.

Genotype	RA Patients N (%)	Controls N (%)	*P*
Total	**200**	**200**	
RP1/RP1	**113 (56.5)**	**0 (0)**	**<0.001**
RP1/RP2	**73 (36.5)**	**40 (20)**	**NS**
RP2/RP2	**14 (7)**	**160 (80)**	**NS**
IL-4 -590 T genotypes			
CC	**23(11.11)**	**150 (75)**	**NS**
CT	**71 (35.56)**	**50 (25)**	**NS**
TT	**106 (53.33)**	**0 (0)**	**<0.001**
IL-4 Rα +1902 A genotypes			
AA	**20 (10)**	**70 (35)**	**NS**
AG	**82 (41)**	**110 (55)**	**NS**
GG	**98 (49)**	**20 (10)**	**NS**
IL-4 Rα +148G genotypes			
AA	**54 (27)**	**80 (40)**	**NS**
AG	**106(53)**	**40 (20)**	**0.02**
GG	**40 (20)**	**80 (40)**	**NS**

** It is significant when p<0.05*

Table 2: Comparison of allelic frequencies and carriage rates observed in rheumatoid arthritis patients.

GENE, ALLELE	Allelic Frequency		Carriage Rate		*P*	OR (95% CI)
	RA Patients No. (%)	Controls No. (%)	RA Patients No. (%)	Controls No. (%)		
RP1 RP2	299(76.1) 101(25)	40 (10) 360(90)	186(94.4) 87(93.3)	40(20) 200(100)	0.04* 0.14	2.91(0.92-10.23) 2.29(0.68-8.54)
-590 C -590 T	117(28.9) 283(71.1)	350(87.5) 50(12.5)	94(46.7) 177(88.9)	200(100) 50(25)	0.15 0.03*	2.1(0.68-6.92) 2.84(1.0-8.77)
+1902 A +1902 G	122(30.5) 262(69.4)	250(62.5) 240(37.5)	102(51.1) 180(90)	180(90) 130(65)	0.39 0.55	1.36(0.63-2.94) 1.27(0.53-3.04)
+148 A +148 G	214(53.3) 186(46.7)	200(50) 200(50)	160(80) 146(73.3)	120(13.3) 120(13.3)	0.84 0.7	1.09(0.42-2.82) 1.14(0.55-2.39)

Table 3: RF titers in relation to IL-4 and IL-4 Rα genotypes.

IL-4, IL-4Rα Genotypes	RF	*P*
RP1/RP1 RP1/RP2 RP2/RP2	81.44±87.64 76.55±82.26 74.33±66.20	>0.05
IL-4 -590 T genotypes CC CT TT	 70.29±9.20 146.00±16.85 272.28±45.57	<0.001
IL-4Rα +1902 A genotypes AA AG GG	 189.73±33.15 87.58±18.57 47.11±11.03	<0.001
IL-4Rα +148G genotypes AA AG GG	 57.56±77.11 69.44±81.99 149.82±73.29	<0.001

Table 4: Association and frequency of the RP1 allele in IL-4 (intron 3) and -590T allele in the IL-4 promoter region in rheumatoid arthritis patients.

Carrier of Allele IL-4 RP1/IL-4 – 590*T	Patients N (%)	Controls N (%)	*P*
+/+	167 (83.5)	0 (0)	0.001<
+/-	11 (5.5)	20 (10)	NS
-/+	20 (10)	30 (15)	NS
-/-	2 (1.11)	150(75)	NS

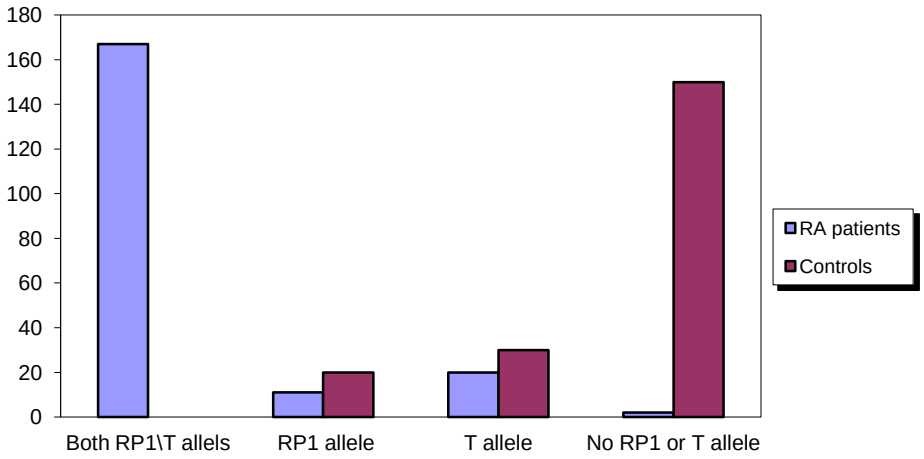

Fig. 5: Relation between IL-4 RP1 allele and IL-4 T allele in RA Patients. Most RA patients (83.33%) are carrying both IL-4 RP1 and IL-4 -590 T alleles where only one patient wasn't carry any of these two alleles.

Our results also showed a significantly higher frequency of the IL-4 intron-3 RP1/RP1 genotype (P <0.001) and IL-4 intron-3 RP1 allele with RA patients compared with controls.

Additionally, a significant association between IL4 -590T allele and IL4 VNTR RP1 allele in RA patients only was seen in our population, a similar result had been reported by Cantagrel et. al. [16].

Our results show a higher frequency of IL-4Rα AG genotypes (at position +148) than AA and GG genotypes among RA patients compared with controls. This is matched with Susan et. al. [24].

Burgos et al. [25], who found an association of IL-4Rα A148G SNPs with rheumatoid nodules in patients with RA. Prots et al [26] also identified the A148G IL4R SNP as a novel genetic marker in RA.

High RF titers were related to IL-4 -590T, IL-4Rα +1902 A and IL-4Rα +148 G allele carriers where the disease is more severe in comparison other alleles in our population.

IgM RF is present in 60–80% of patients with RA and the titer correlates with disease severity and extraarticular manifestations [27]. Patients with high RF titers have articular damage more persistent and severe than those with low titers or negative RF [28].

Our results are in good agreement with Moreno et al [29], who found that IL4 –590TT genotype was significantly more frequent in patients with RA than in controls.

The experiments indicated a 3-fold higher promoter activity of the T allele and enhanced IgE production [30]. It has been shown that C-to-T substitution at position -590 relative to the transcription start site creates the changed core sequence of the nuclear factor of activated T cells (NFAT) contact site and it is possible that there is a linkage disequilibrium between the IL4 -590 T allele and other genes that are important in the control of the immune response.

The human gene for IL-4 has been mapped to the Q arm of chromosome 5 in a cluster of cytokine genes (IL-3, IL-5, IL-9, IL-13 and IL-15) [4]. Although the -590 T allele is associated with enhanced promoter activity and probably increased IL-4 production, nevertheless, the linkage disequilibrium with other genes that are responsible for the transcriptional activation of proinflammatory factors may cause the increased susceptibility to RA and greater disease severity [31].

Suzan et al. [24] indicated that an inherited polymorphism of the IL-4R controls the ability of the human immune system to regulate the magnitude of IL-17 production. However, in established RA, this pattern may be altered, possibly due to secondary effects of both RA itself as well as immunomodulatory medications. Ineffective control of Th1 immune responses is a potential mechanism to explain why IL-4R is an important severity gene in RA.

Conclusion

Evidence is accumulating that IL-4 has a complex function in the development of innate and adaptive immunity and might play an important role in the preclinical or early phases of RA. Our results show an association of IL4 –590T, IL-4RP1 alleles and IL-4Rα A148G genotype with RA susceptibility. The IL-4 –590T, IL-4 Rα G148 and IL-4 Rα A1902 allele with RA severity.

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