

Detection of the -1082 G/A Promoter Polymorphism of Interleukin-10 Gene by ARMS-PCR Technique among some Iraqi Rheumatoid Arthritis Patients

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease in which interleukin (IL)-10 gene plays an important role. There are, however, controversial reports that IL-10 promoter polymorphism may be an independent marker of susceptibility and severity of RA. The aim of the present study was to examine the IL-10 promoter polymorphism among RA patients.

The aim of this study was to investigate the association between IL-10 G-1082A promoter polymorphism of interleukin-10 gene in some Iraqi rheumatoid arthritis patients using ARMS-PCR technique.

We examined 70 RA patients (60 females and 10 males; aged: 20 – 74 years, mean: 51.7 years) were recruited from a private clinic in different region of Salahaddin governorate and 50 healthy individuals (40 females and 10 males; age: matching with patients) who were the staff of Tikrit University and hospital workers. The venous blood samples (5 ml) were collected from all the participants at the time of clinical examination, aliquoted in plain and EDTA vacutainers for the determination of erythrocyte sedimentation rate, C-reactive protein, presence of RFs. Genomic DNA was isolated from the whole-blood samples of all the patients and control subjects. The IL-10 -1082 G/A polymorphism genotyping Amplification Refractory Mutation System- Polymerase Chain Reaction (ARMS-PCR) method was used.

ESR rate and RF positivity Levels of CRP were found as high as ≥ 20 , 86.3% among patients, while all the healthy individuals were negative for this test except for a few number.

The genotype GA for the -1082 G/A polymorphism of the IL-10 gene promoter was the most frequently observed genotype in both patients and controls, however, the distribution of the GG and AA genotype in patients and control was different. While genotype AA has a higher frequency than GG in patients (27.04vs.23.04), genotype GG was more frequently observed than AA in controls (46.08vs. 0.08).

Keywords: Interleukin-10 Promoter polymorphism, Rheumatoid arthritis, ARMS-PCR

ARMS-

IL-10

1082 G/A

PCR

(IL-10) 10

MATERIALS AND METHODS

Subjects

The studied samples consisted of 70 RA patients (60 females and 10 males; aged: 20 – 74 years, mean: 51.7 years) were recruited from a private clinic in different regions of Salahaddin governorate during the period from (December 2012 – February 2013) and 50 healthy individuals (40 females and 10 males; age: matching with patients) who were staff of Tikrit University and hospital workers. The diagnosis of RA was made in accordance with the criteria of the American College of Rheumatology (ACR) modified in 1987, information regarding the demographic features such as age, marital history, parity, gestational age, family history, consanguinity, etc was obtained from all these subjects with the help of a standard perform questionnaire.

Sample Collection

The venous blood samples (5 ml) were collected from all the participants at the time of clinical examination, aliquoted in plain and EDTA vacationers for the determination of erythrocyte sedimentation rate, C-reactive protein and presence of RFs. The remainder of blood samples was stored at -20°C for further molecular analysis.

DNA Extraction

Genomic DNA was isolated from the blood samples of all the patients and control subjects using a method described by (Al-azawy,2012). DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 and 280 nm using (Nano drop Thermo scientific, Germany) and detected the optimum DNA concentration for ARMS-PCR analysis. The quality of the DNA was determined using agarose gel electrophoresis stained with ethidium bromide, samples were stored at -20 °C until further use.

Determination of IL-10-1082G/A polymorphism by ARMS-PCR:

For IL-10 -1082 G/A polymorphism genotyping Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method was used as described by Bhayal *et al.*, (2012). For each polymorphism two separate reactions were performed. Each reaction contained one of the two-allele specific forward primers and a generic anti sense primer. The primers used are as follows: a common (CF) anti-sense primer 5'-GTA AGC TTC TGT GGC TGG AGT C-3'; (FG) Sense 5'- AAC ACT ACT AAG GCT TCT TTG GGT G-3'G-primer; (FA) Sense 5'-AAC ACT ACT AAG GCT TCT TTG GGT A-3'. These reactions amplify allele specific sequence of 161 bp of the promoter of IL-10 gene. The PCR amplification was performed in a total volume of 25 µl. 12.5 µL of Go Taq green master mix 2X (green master mix is a premixed ready to use solution containing Taq DNA polymerase, dNTPs, MgCl₂ and reaction buffers) supplied by promega company (USA), 2 µL (100 ng) of genomic DNA, 1µl of each primer (10 pmol/1µL) and made up to 25 µL with nucleases free water. The thermal cycling was as follows: Initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 63°C for 30s, and 72 °C for 30s, one cycle. The final extension at 72 °C for 5 min. The amplified PCR products (161bp) were separated by 1.5% agarose gel electrophoresis in the presence of 1Kb DNA ladder (Biolabs-England) as a molecular size marker, and visualized using a gel documentation System after ethidiumbromide staining (10 mg/ml).

Statistical analysis

The allelic and genotype frequencies of the IL-10 gene in the control and case groups were calculated by direct count. Chi-square (χ^2) test was used to compare allele/genotype frequencies between groups, and Hard-Weinberg equilibrium of genotype distribution.

RESULTS AND DISCUSSION

Age and gender distribution of patients and control is listed in table-1. The higher frequencies of disease were observed at third (24%) and fourth decade (84%) respectively. A higher incidence was noticed among the females (74.0%) rather than the males; in 2.8:1 ratio.

Table 1: Distribution of Rheumatoid Arthritis patients by age and gender in comparison with healthy samples

Age Groups (Year)	Studying sample			
	RA. Patients		Healthy sample	
	No.	%	No.	%
< 20	8	8	8	16
20-29	9	9	9	18
30-39	*24	24	19	38
40-49	17	17	8	16
50-59	20	20	4	8
60-69	16	16	2	4
70-79	6	6	-	-
Female	*84	84	34	71
Male	16	16	16	29
Total	100	100	50	100

* Higher frequency

The demographic features of the RA Iraqi patients in addition to the healthy normal individuals are listed in Table (2).

Table 2: Clinical and demographic parameters of RA patients and healthy samples

Disease in years	RA Patients	Healthy sample
Age of subjects	20 – 60 Year	20 – 60 Year
1 – 15 Year		-
Age of RA onset years	18	-
ESR	≥ 20	≤ 10
RF test positivity %	78	7
CRP test positivity %	90	5

The average of the patient's age was between 18-79 years where as the average of the age of the healthy control group was between 17-67 years. ESR rate and RF positivity Levels of CRP were found as high as ≥ 20 , 86.3% among patients, while all the healthy individuals were negative for this test except for a few number.

Based on the above definition of the genotype for the -1082 G/A polymorphism of the IL-10 gene promoter, the genotype distributions and allele frequencies of the SNP were counted, calculated and summarized in Table (3). The genotype frequencies of the -1082 G/A SNP in RA patient and normal control groups were all in accordance with the Hardy-Weinberg equilibrium (data not shown, P value 0.01).

Table 3: Distribution of RA patients and healthy samples according to Allele frequency and genotyping

Group	No.	Allele	Allele Frequency	Genotype	Expected No.	Observed No.	χ^2	P value
RA Patients	100	G	0.48	GG	11	23.04	23.27	0.01
		A	0.52	GA	74	49.92		
		AA	15	27.04				
Healthy Samples	50	G	0.96	GG	46	46.08	0.087	0.01
		A	0.04	GA	4	3.84		
		AA	0	0.08				

The genotype GA for the -1082 G/A polymorphism of the IL-10 gene promoter was the most frequently observed genotype in both patients and controls (74 and 46 respectively). However, the

distribution of the GG and AA genotype in patients and control was different. While genotype AA has a higher frequency than GG in patients (27.04vs.23.04), genotype GG was more frequently observed than AA in controls (46.08vs. 0.08). Further calculation of allelic frequency indicated a higher frequency of A than that of G in patients (0.52vs. 0.48). In contrast, a lower frequency of A allele than G allele in the control group (0.04vs. 0.96). Chi-square analysis was performed to compare the -1082 G/A polymorphism of the IL-10 gene promoter genotype distribution and allele frequency in patient and control groups, and it was found that both genotype and allele distribution were significantly difference in the two groups. The calculated chi-square of the patient's group was to be 23.27 more than χ^2 tabulated (6.63), so there are significantly different between genotypes of RA patients ($P=0.01$). In case of controls, no significant association was found for controls ($P=0.01$) and calculated chi-square was found to be 0.08 less than χ^2 tabulated (6.63). The results suggest that allele A is significantly associated with RA.

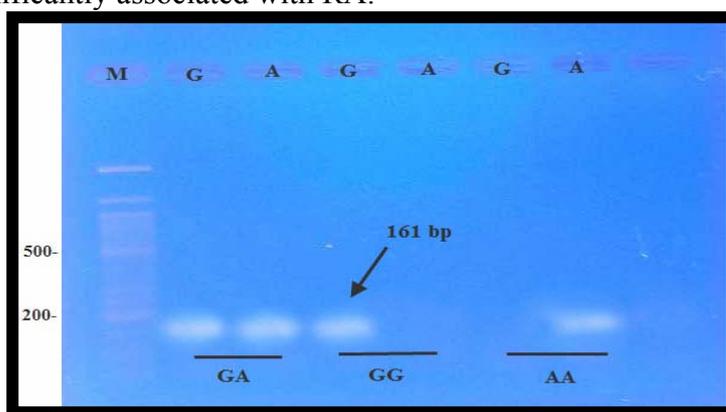


Fig. 1: A representative agarose (1.5%) gel electrophoresis of ARMS-PCR products for IL-10-1082 G/A polymorphisms. M: 100bp size DNA ladder.

Several studies examined the role of the association of IL-10 promoter polymorphisms for RA susceptibility and severity (Andrzej *et al.*, 2005 ; Fei *et al.*, 2012). Padyukov *et al.*, (2004) showed that -1082 AA genotype frequency in women with RA was significantly lower than controls. Pawlik *et al.*, (2005) found that -1082 GG allele and GCC/GCC haplotypes frequency were significantly higher than controls. Hajeer *et al.*, (1998) found no significant difference in allele frequencies of IL-10 polymorphisms between RA patients and controls, but they showed that the genotypes encoding low IL-10 gene expression correlated with IgA rheumatoid factor seropositive RA (Hajeer *et al.*, 1998). Three further studies also failed to show any association between IL-10 polymorphism and RA (Omar *et al.*, 2008).

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