Investigation of the Genetic Polymorphism Of Interleukin-10
Gene in Rheumatoid Arthritis Patients in Egypt


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Abstract: Rheumatoid arthritis is an inflammatory and considerably variable disease with both genetic and environmental factors contributing to the susceptibility to it as well as its severity. Two groups of Egyptian subjects were included; one group consisted of 54 clinically confirmed rheumatoid arthritis patients (Group I) and the other group consisted of 24 apparently healthy individuals (Group II). DNA was isolated from peripheral blood cells of both groups and genotyped by RFLP technique on IL-10 gene promoter. For rheumatoid arthritis patients, 5.5% were of the genotype AA and 40.7% were heterozygous (CA) while the remaining 53.7% were homozygous for the allele C. On the other hand, for the apparently healthy individuals, 15.3% were of the genotype AA and 15.3% were heterozygous (CA) while the remaining 61% were homozygous for the allele C. Statistically, there was a significant difference in the distribution of the genotype CA between rheumatoid arthritis patients and the apparently healthy individuals. This result suggests that the subjects with the genotype CA could be at more risk for developing rheumatoid arthritis than the subjects with other genotypes. This risk could not be attributed to the carriage of either allele A or C solely because the frequencies of A allele and C allele were not significantly different between the rheumatoid arthritis patients and apparently healthy individuals. Whether the -592 CA genotype of IL-10 implies a moderate IL-10 expression and a low efficiency to reduce inflammation in Egyptian subjects with rheumatoid arthritis needs further investigations.

Key words: IL-10 · Cytokine · Polymorphism · Susceptibility · Rheumatoid Arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory and considerably variable disease and both genetic and environmental factors can contribute to susceptibility to disease and the disease severity [1]. Cytokines are involved in the pathogenesis of RA and, therefore, their polymorphic genes are considered as potential markers of RA severity [2] and they can be considered as risk factors for RA [3]. The genetic susceptibility to RA was investigated in several studies [3-7] and the genetic factors were considered to be strong determinants of RA. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines and a potent up-regulator of B-cell production and differentiation [8]. In animal model, IL-10 modulates disease severity of RA through suppressing joint swelling and deformation and cartilage necrosis [9]. In human, IL-10 is up regulated in the serum and synovial fluid of RA patients [9]. The production of IL-10 is thought to be genetically controlled during gene transcription via some regulatory sequences in its promoter region [10].

IL-10 production is known to be under strong genetic influence [11] and there are some evidences about the association of single nucleotide polymorphisms (SNPs) in the IL-10 gene promoter and the differential expression of IL-10 in vitro[12]. This study aimed to check whether there is an association between the IL-10 gene promoter polymorphism at the position-592 and rheumatoid arthritis in the Egyptian patients.
MATERIALS AND METHODS

Blood Sampling and DNA Extraction: Peripheral blood samples were collected in EDTA-rinsed vacutainer tubes by venipuncture from the arm vein of 54 clinically confirmed-rheumatoid arthritis subjects (Group I) and 24 apparently healthy individuals (Group II) in Mansoura University Hospital after providing informed consent.

Genomic DNA was extracted from all blood samples by Gene JETTM Genomic DNA Purification Kit (#K0722) according to the manufacturer’s instructions. The purified DNA was immediately used in downstream applications or stored at -20°C.

Polymerase Chain Reaction (PCR): All DNA samples extracted from group I and group II were subjected to PCR to amplify a part of IL-10 gene promoter region using the forward primer 5’-GGTGAGCACTACCTGACTAGC-3’ and the reverse primer 5’-CTAGGTCAACATGACGCTG-3’[13]. Primers were synthesized in Pioneer Corporation (South Korea). All PCR reactions included 25 µl of Maxima Hot Start PCR master mix kit (#K1051, Fermentas, Germany), 2 µl of the forward primer and 2 µl of the reverse primer and 4 µl of each DNA sample were added to individual PCR mixtures and the reaction was brought to a final volume of 50 µl with nuclease-free water. Reactions were loaded to a thermal cycler (Multigene, Labnet, USA) programmed as follows: 4 min at 95 ºC for initial enzyme activation followed by 40 cycles of 30 sec at 95 ºC for denaturation, 30 sec at the optimized temperature for annealing and 1-2 min at 72 ºC for extension and a final extension step for 10 min at 72 ºC. PCR products were loaded with 100 bp DNA ladder (#SM0323, Fermentas) on 1.5% agarose gels for 40 min in 1x Tris/Borate/EDTA (TBE) buffer. Gels were stained for 20 min with Ethidium Bromide (Bioshop, Canada) and photographed in a gel documentation system (Photo Doc-IT Imaging system, USA).

Restriction Digestion: PCR products amplified from the DNA samples of 54 RA group I and group II subjects were digested with Rsal restriction Enzyme kit (#ER1121, Fermentas) in a 30 µl scale reaction containing 10 µl of the PCR reaction mixture, 2 µl of the reaction buffer R, 2 µl of Rsal restriction enzyme (20 units) and 16 µl of nuclease-free water. Restriction reactions were loaded side by side with an equivalent amount of PCR reaction mixture in 2% agarose gels for 40 min in TBE buffer. Gels were stained and photographed as mentioned above.

RESULTS

Genotype Frequencies: To investigate the association of IL10 gene polymorphism with the risk of rheumatoid arthritis, we used RFLP technique to study the C to A transition at the position –592. DNA was extracted from the blood of 54 patients (Group I, clinically confirmed rheumatoid arthritis patients) and 24 subjects (Group II, apparently healthy individuals) and the quality of the extracted DNA was confirmed by gel electrophoresis (Not shown). The region spanning the nucleotides -803 to -391 of the IL10 gene promoter region was amplified by PCR which resulted in a 412bp product expected (Fig. 1, lanes 1 to 5). The amplified PCR products from all subjects were digested with the restriction enzyme Rsal and the restriction digestion reactions were run parallel to the undigested PCR products on the same gels (Fig. 1, lanes R in rows A-E). Rsal enzyme has partially cleaved the PCR products into 2 fragments; 176bp and 236bp approximately in 25 clinically confirmed rheumatoid arthritis patients. To confirm whether this partial digestion is due to the heterozygous nature of these DNA samples or because of excess of DNA in the digestion reactions, PCR products from these 25 DNA samples were digested again at the same conditions but using smaller amounts of the PCR products (Fig. 2). This digestion reaction resulted in a complete digestion of 3 samples (9, 13 and 26) and the partial digestion of the other 22 samples. Altogether, these results indicate that 3 patients were of the genotype AA and 4 patients were CA while the other 16 patients are CC. Statistically, there was a significant difference in the distribution of the CA genotype between the clinically confirmed rheumatoid arthritis patients (40.7%) and the apparently healthy individuals (16.67%) (Table 1).

Distribution of the three genotypes was statistically significant within Group I (p=0.001) and also within Group II (p=0.002) (Table 2). The homozygous CC genotype was detected in 53.7% (29 out of 54) of the subjects with rheumatoid arthritis (Group I) and in 61% (16 out of 24) of
Fig. 1: PCR and restriction digestion of DNA samples extracted from the blood of 54 clinically confirmed RA patients.

Fig. 2: Confirmation of the restriction digestion pattern of the 29 partially digested DNA samples from clinically confirmed RA patients.

Fig. 3: PCR and restriction digestion of DNA samples extracted from the blood of 24 apparently healthy patients.
Table 1: Difference in the distribution of IL-10 genotypes between clinically confirmed Rheumatoid Arthritis patients (Group 1) and apparently healthy subjects (Group 2).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group 1 (n=54)</th>
<th>Group 2 (n=24)</th>
<th>( \chi^2 )</th>
<th>Significance(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>29 (53.7%)</td>
<td>16 (66.67%)</td>
<td></td>
<td>0.285</td>
</tr>
<tr>
<td>CA</td>
<td>22 (40.7%)*</td>
<td>4 (16.67%)</td>
<td></td>
<td>0.037</td>
</tr>
<tr>
<td>AA</td>
<td>3 (5.5%)</td>
<td>4 (16.67%)</td>
<td></td>
<td>0.113</td>
</tr>
</tbody>
</table>

*, refers to statistical difference at 0.05.

Table 2: Distribution of IL-10 genotypes among clinically confirmed Rheumatoid Arthritis patients (Group 1) and among the apparently healthy subjects (Group 2).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group 1 (n=54)</th>
<th>Group 2 (n=24)</th>
<th>%</th>
<th>sig. (p)</th>
<th>No.</th>
<th>%</th>
<th>sig. (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>29</td>
<td>16</td>
<td>53.70%</td>
<td>0.001</td>
<td>16</td>
<td>66.67%</td>
<td>0.002</td>
</tr>
<tr>
<td>CA</td>
<td>22</td>
<td>4</td>
<td>40.74%</td>
<td></td>
<td>4</td>
<td>16.67%</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>3</td>
<td>4</td>
<td>5.56%</td>
<td></td>
<td>4</td>
<td>16.67%</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Number and percentage of subjects carrying any allele C or carrying any allele A in Group I (Rheumatoid Arthritis patients) and Group II (apparently healthy subjects).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group 1 (n=54)</th>
<th>Group 2 (n=24)</th>
<th>%</th>
<th>%</th>
<th>( \chi^2 )</th>
<th>Significance(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any allele C (CC or CA)</td>
<td>51 (94.4%)</td>
<td>20 (76.3%)</td>
<td>0.52</td>
<td>0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any allele A (CA or A)</td>
<td>25 (46.2%)</td>
<td>8 (30.6%)</td>
<td>0.36</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

the apparently healthy individuals (Group II). 5.5% of subjects (3 out of 54) in Group I and 15.3% of subjects (4 out of 24) in Group II were homozygous for the allele A. Heterozygous genotype (CA) was detected in 40.7% (22 out 54) of Group I and in 15.3% (4 out of 24) of Group II.

**Frequencies of Allele Carriage:** The frequencies of C allele carriage, i.e. the proportion of subjects in the two groups carrying at least one C allele, are reported in table 3. No statistical differences \((p=0.52)\) were found between the entire Group I (n=54) and Group II (n=24). The frequencies of A allele carriage, i.e. the proportion of subjects in the two groups carrying at least one A allele, are also reported in table 3. No statistical differences \((p=0.36)\) were found between the entire Group I and Group II for either A allele or C allele carriage.

**DISCUSSION**

Paleopathological studies of Egyptian mummies suggest the existence of RA in Egyptians. Based on his studies, G. Elliot concluded that RA was par excellence the disease of Egyptians, however, many authorities do not agree and consider RA to be a modern disease [14].

Up to the best of our knowledge and based on the published literature, so far there are no studies addressing the association between Rheumatoid arthritis and the polymorphism in the IL10 gene in Egyptian patients. Therefore, this study was undertaken to investigate the C to A transition at the position-592 of IL10 gene promoter in rheumatoid arthritis patients in Egypt.

The data presented here indicated that, for the rheumatoid arthritis patients, 5.5% were of the genotype AA and 40.7% were heterozygous (CA) while the remaining 53.7% were homozygous for the allele C. On the other hand, for the apparently healthy individuals, 15.3% were of the genotype AA and 15.3% were heterozygous (CA) while the remaining 61% were homozygous for the allele C. Chi-square analysis revealed a significant difference in the distribution of the genotype CA between rheumatoid arthritis patients and the apparently healthy individuals. This result suggests that the subjects with the genotype CA could be at more risk for developing rheumatoid arthritis than subjects with the other genotypes. According to the data presented here, this risk can not be attributed to the carriage of either allele A or C solely because the frequencies of A allele carriage in the rheumatoid arthritis patients (AA or CA) were not significantly different from the frequencies of A allele carriage in the rheumatoid arthritis patients and, also, there were no difference in frequency of C allele carriage.
The association between IL-10 polymorphisms and RA has been reported in some previous studies elsewhere in the world. In a study by Lee et al. [15] about the significance of IL-10 gene polymorphism (-1082 G/A,-819 C/T and -592 C/A), the Meta-analysis of the IL-10 -592 C/A polymorphism revealed a significant association with RA in Asians and the C allele carriage was suggested to be a protective factor. Huizinga et al. [9] found an association between the -1082 G/A polymorphism and the rate of radiographic damage in a longitudinally followed cohort of female RA patients.

This kind of association was also reported in other kind of diseases. Wu et al. [16] reported that the IL-10 -592 CA genotype was associated with the susceptibility of developing child patients with chronic immune thrombocytopenia, but the IL-10 gene polymorphisms was not associated with the susceptibility of adult patients with chronic immunothrombocytopenia. A similar association for IL-10 -819 C/T and IL-10 -592 A/C and coronary artery disease at 5% significance level was observed in a study of 1060 low- or high-risk men [17]. The -592 allele A of II-10 was found to be associated with coronary events in a study on 5804 subjects in Scotland, Ireland and Netherlands [18].

On the other side, according to Lee et al. [15], combined data from published studies about genetic associations between the polymorphisms-1082 G/A,-592 C/A,-892 C/T of IL-10 showed no association with RA for several subjects, however, the meta-analysis of the-1082 G allele in some cases revealed association with RA. In a quantitative assessment for the association between IL-10 gene -592 C/A polymorphism and risk of Type 2 Diabetes mellitus(T2DM), Yin et al. [19] found that the IL-10 gene -592 C/A polymorphism is not associated with T2DM risk. No association was found between IL-10 alleles of the-1082 G/A,-819 C/T and-592 C/A polymorphisms, haplotypes/genotypes and RA [20].

Interleukin-10 (IL-10) is a major immunomodulatory cytokine that is thought to mediate down regulation of the inflammatory response in RA and is suggested to play an important role in the pathogenesis of joint destruction in RA [2].

IL-10 is produced mainly by T helper 2 (Th2) lymphocytes, B lymphocytes and also by macrophages and is present in higher concentrations in serum and synovial fluid from RA patients [2].

Polymorphisms at the positions-1082G/A,-819C/T and -592C/A of the IL-10 gene have been reported to affect the production of IL-10 and lower IL-10 expression has been reported in patients with the-592AA and-1082AA polymorphisms (low IL-10-producer groups) in many studies [11]. In addition, most publications report that the ATA/ATA IL-10 haplotype (-1082A/A,-819T/T and-592A/A) is associated with the lowest levels of IL-10 expression, while the GCC/GCC haplotype (-1082G/G,-819C/C and-592C/C) is associated with the highest levels of IL-10 expression [21-26].

Theoretically and based on the finding that individuals with the-1087G allele or GG genotype are higher producers of IL-10 [27, 28], such individuals might exhibit a normal level of IL-10 that would reduce inflammation and bring the situation into homeostasis [12].

Whether the-592 AC genotype of IL-10 in the current study implies a moderate expression of II-10 that is inefficient to reduce inflammation in Egyptian subjects with Rheumatoid arthritis needs further investigations. Considering the complex etiology of RA, the exact mechanisms that control the disease development must be addressed more.

To the best of our knowledge, this is the first study to date to investigate the association between IL-10 gene polymorphism and RA risk in Egyptian patients.

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REFERENCES


