GENETIC POLYMORPHISM IN PROTEIN TYROSINE PHOSPHATASE NON-RECEPTOR 22 (PTPN22) GENE IN IRAQI PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation and joint destruction. The aim of the present study was to investigate the genetic polymorphism (rs2746601) for the gene encoding protein tyrosine phosphatase non-receptor 22 (PTPN22) gene in Iraqi RA patients. A total of 35 samples (25 RA patients and 10 healthy controls) were recruited in this study. Genetic polymorphism in Protein Tyrosine Phosphatase non-receptor 22 (PTPN22) was studied for rs2476601 SNP in exon 14 within chromosome 1. This SNP (rs2476601) was regarded as a major risk factor associated with susceptibility and severity of rheumatoid arthritis. Genomic DNA was first extracted from blood samples for healthy controls and treated RA patients. Results showed that the concentration of extracted DNA ranged between 100-200 ng/μl with purity of 1.8-2.0. Then exon 14 was amplified by using specific primers designed in this study. Results of amplification showed a single band of 684bp was obtained after electrophoresis on agarose gel (1.8%) which represents the complete nucleotide sequence of exon 14. To examine genetic polymorphism (rs2476601) in this exon, the nucleotide sequence for this fragment was determined. Results showed that the rs2476601 SNP was non-polymorphic in both RA patients and healthy control subjects with total absence of the variant ‘T’ allele. Furthermore, the frequency of the ‘T’ allele was 0.0, with T/T, C/T and C/C genotype frequencies of 0.0, 0.0, and 1.0, respectively. In conclusion, this
study shows that the rs2476601 SNP of the PTPN22 gene is non-polymorphic in Iraqi population and therefore not associated with RA. However, since variations in the rest of the gene were not investigated, these results do not exclude other PTPN22 polymorphisms from playing a role in RA susceptibility in Iraq.

INTRODUCTION
Rheumatoid arthritis is a chronic inflammatory autoimmune disease characterized by synovial inflammation and structural damage of joints[1]. The diagnosis of rheumatoid arthritis is based on clinical manifestations of joints and serological markers[2]. Rheumatoid arthritis patients are characterized by presenting some circulating auto antibodies in their serum. In clinical practice the most common diagnostic test is rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) and is generally accepted by the majority of rheumatologists and recommended by the European League of Arthritis and Rheumatism (EULAR)[3].

The etiology of RA, like other autoimmune disorders are not fully understood[4]. However, RA is complex disorder that interactions of genetic and environmental factor contribute to susceptibility[5]. Genetic factors contribute 50% to 60% of the risk of developing RA. The major genetic risk factors that have been reproducibly shown strong association with susceptibility are Human leukocyte antigen of DRB1 alleles Human leukocyte antigen of DRB1 alleles (HLA-DRB1), and Protein tyrosine phosphatases non- receptor 22 (PTPN22) genes[6][7]. Among non-HLA genes, the main RA susceptibility factor is the tyrosine-phosphatase gene PTPN22 on chromosome.[8] A missense C→T substitution at nucleotide position 1858 (rs2476601) of this gene leads to substitution of tryptophan (W) for arginine (R) at residue 620 of the protein product.[9] The resulting gain of function, with enhanced regulation of T-cell receptor (TCR) signaling during thymic selection, permits autoantigen specific T cells to escape clonal deletion, thereby predisposing to autoimmunity.[10] This PTPN22 polymorphism is not seen in Asian populations, may be only with Asiatic Indians with RA positive.[11]

MATERIALS AND METHODS
Subjects
Blood samples (3-5 ml) were collected in EDTA anticoagulant tubes from 25 male and female patients with Rheumatoid arthritis from Rheumatology consultation clinic in Baghdad
Teaching Hospital through the period from November 2014 to June 2015. Another blood samples were collected from 10 apparently healthy controls.

**Extraction of Genomic DNA**

Total genomic DNA was extracted from blood samples by using ReliaPrep™ Blood gDNA Miniprep System supplied by Promega Company (USA).

**Amplification of Protein Tyrosine Phosphatase Non-receptor 22 Exon 14**

Amplification of *PTPN22* was achieved by using specific primers indicated in (Table 1). These primers were designed in this study and were supplied by Alpha DNA Company in a lyophilized form of different picomols concentrations. Lyophilized primers were dissolved in a free DNase/RNase water to give a final concentration of 100 pmol/μl as a stock solution. A working solution of 10 pmol/μl of these primers was prepared by adding 10μl of primer stock solution to 90μl of deionized distilled water to get a final concentration of 10 pmol/μl.

**Table 1: Oligonucleotide primers used for the amplification of *PTPN22* Exon 14**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→ 3')</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>AAGAATAAGCAAAAAACCTCCTGGG</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>AACATTGAAAGGACCTGAGAAGT</td>
<td>684</td>
</tr>
</tbody>
</table>

PCR amplification program for Exon 14 of *PTPN22* was achieved by initial denaturation for one cycle at 95 °C for 4min., followed by 30 cycles of denaturation at 95 °C for 30sec, annealing at 60 °C for 30sec, extension at 72 °C for 30sec, and the final extension for one cycle at 72 °C for 7min.

**Sequencing of amplification products**

Amplification products for exon 14 of the gene *PTPN22* was sent for sequencing to Macrogen Company (USA). Then, the sequencing for these products was compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard *PTPN22* gene, using (Bioedit) software.

**RESULTS AND DISCUSSIONS**

Genomic DNA was first extracted from blood samples of patients and healthy controls by using ReliaPrep™ Blood gDNA Miniprep System (Promega / USA). The concentration of DNA solutions extracted from all blood samples was ranged between 100-200 ng/μl, while
the purity was ranged between 1.8-2.0. This purity and concentration of DNA solutions were suitable and recommended for further genetic analysis by using PCR technique[12].

Polymerase chain reaction was used to amplify exon 14 of PTPN22 in the extracted genomic DNA under optimum amplification conditions by using specific primers shown previously. Results illustrated in (Figure 1) showed a single band of the amplified product with a molecular size of 684 base pair after electrophoresis on agarose gel (1.8%). These DNA fragments represent the region of exon 14 for PTPN22 gene located on chromosome 1p13[13].

![Figure 1](image_url)

**Figure 1:** Gel electrophoresis for PCR products of PTPN22 gene exon 14 on agarose gel (1.8%) after electrophoresis for 1 hour at 5 v/cm² in the presence of 1 kb DNA Ladder marker. (A) PCR products for DNA extracted from blood samples of patients with Rheumatoid arthritis. (B) PCR products for DNA extracted from blood samples of healthy controls.
Sequencing of Amplified Exon 14 of PTPN22 Gene

In this study, twenty five Iraqi patients with rheumatoid arthritis were recruited to investigate the relationship between rs2476601 SNP in exon 14 of PTPN22 gene and the severity of the disease compared with ten Iraqi healthy controls.

Protein Tyrosine Phosphatase (PTPN22) gene was the first non-HLA gene that associated with rheumatoid arthritis[14]. PTPN22 encodes the lymphoid tyrosine phosphatase (LYP) that modulates the activation of both T and B cells [15]. Single nucleotide polymorphisms (SNPs) in PTPN22 gene (rs2476601) are highly associated with the disease in several ethnic populations[16], and regarded as the most important risk factor associated with the susceptibility of RA[17]. In the European population, the second strongest genetic risk outside of the HLA region is a functional single nucleotide polymorphism (SNP) in the PTPN22 gene which is a C→T substitution at nucleotide position 1858 (rs2476601), which results in a substitution of tryptophan (W) for arginine (R) at codon 620[10]. However, the rs2476601 SNP was not found to be associated with RA in the Asian population[18][19].

Nucleotide sequence of exon 14 was analyzed by blastn in the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit program to detect rs2476601 polymorphism in exon 14 of PTPN22 gene. Results illustrated in (Figure 2) showed the nucleotide sequence alignment and the position of the cytosine (C) nucleotide of healthy control group matched with cytosine (C) nucleotide in reference sequence mentioned in NCBI, that showed 100% identity, under Sequence ID: gb|EF064714.1| from 38554-39158 number of nucleotide from Homo sapiens protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22) gene, from Gene Bank, with score (1118) and expect 0.0.

On the other hand, results of sequencing of PTPN22 exon 14 in Iraqi patient with rheumatoid arthritis illustrated in (Figure 3) and (Table 2) that showed that nucleotide alignment of RA patients group compared with reference sequence mentioned in NCBI, with 100% identity, under sequence ID: ref|NG_011432.1| from 41613-42218 number of nucleotide from Homo sapiens Protein Tyrosine Phosphatase, non-receptor type 22 (PTPN22), Ref Seq Gene on chromosome 1.

Homo sapiens protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22) gene, Sequence ID: gb|EF064714.1|. 
Figure 2: Alignment sequence of *PTPN22* gene exon 14 of 10 healthy control samples compared with standard *PTPN22* gene exon 14, obtained from Gene Bank. Query represents of samples; Sbject represent of database of National Center Biotechnology Information (NCBI). Yellow letters indicate the position of expected SNP.

*Homo sapiens* protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (*PTPN22*) gene, Sequence ID: ref|NG_011432.1|
Figure 3: Alignment sequence of PTPN22 gene exon 14 of 25 RA patient samples compared with standard PTPN22 gene exon 14, obtained from Gene Bank. Query represents of samples; Sbjct represent of database of National Center Biotechnology Information (NCBI). Yellow letters indicate the position of expected SNP.

Table 2: Sequencing ID in Gene Bank, Score, Expect, and Compatibility of DNA sequences for PTPN22 gene.

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>Accession</th>
<th>Compatibility</th>
<th>Expect</th>
<th>Score</th>
<th>Number of nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 Controls</td>
<td>gb</td>
<td>EF064714.1</td>
<td>100 %</td>
<td>0.0</td>
<td>1118</td>
</tr>
<tr>
<td>25 Patients</td>
<td>ref</td>
<td>NG_011432.1</td>
<td>100 %</td>
<td>0.0</td>
<td>1120</td>
</tr>
</tbody>
</table>
In total, 35 samples (25 RA patients and 10 healthy controls) were analyzed for the rs2476601 of PTPN22 gene. Results of analyzed sequence showed that all samples were genotyped as homozygous CC (Figure 4) and (Table 3).

Figure 4: Sequencing result for rs2476601. Chromatogram shows homozygote CC genotype for rs2476601 of PTPN22 gene. A: Healthy Control group, B: RA Patient group.

Table 3: Genotypes and allele frequency in patients and healthy controls.

<table>
<thead>
<tr>
<th>Samples</th>
<th>N</th>
<th>Genotypes</th>
<th>Alleles (Frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>C/T</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>RA Patients</td>
<td>25</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

N= Number of subject studied

In this study, the role of expected missense SNP (rs2476601) in a gene encoding PTPN22 was studied for its role in RA disease susceptibility and severity in Iraq population. Results indicated in (Table 3) showed that the genetic variant in the position of the expected SNP (rs2476601) was found to be non-polymorphic in all Iraqi population under study. These results agree with those obtained by Mastana, et al, and Somayeh, et al, who found that the rs2476601was non-polymorphic and play no role in susceptibility to RA and other autoimmune diseases in Asian Iranian population, respectively [11][16]. Song, et al, showed that PTPN22 polymorphism (rs2476601) confers susceptibility to RA in populations with different ethnicities, especially in European [20]. However, this does not mean that functional
polymorphisms of the \textit{PTPN22}, other than rs247660 do not play a role in the incidence of RA in Iraqi population. In the Japanese, \textit{PTPN22} allelic heterogeneity was studied, but none of the 8 other SNPs present in the European populations, were found to be polymorphic\cite{18}.

Another explanation may be that the variants in the \textit{PTPN22} gene may reflect genuine differences in the pathogenesis of RA between European and Iraqi patients. However, this can only be said with certainty once all variants of the gene have been studied. Also, this study shows further evidence of inter-ethnic genetic variability in RA at non-HLA loci in Iraq population. Such inconsistent associations might reflect differences in sample size, although this is unlikely in this study as the rs2476601 was non-polymorphic in both RA patients and controls. These differences might also relate to clinical phenotypic differences between populations\cite{21}.

In conclusion, the presence of imbalance of cytokine production oxidative stress in the absence of the \textit{PTPN22} high risk allele of SNP (rs2476601), demonstrates the possible role of these parameters in the pathogenesis of joint damage in Iraqi patients with RA. Furthermore, the possibility of the role of other genetic variants, not yet identified, in Iraqi RA patients. The findings show conclusively that the functional R620W variant, a major risk factor for RA in European populations, is not present and therefore does not play a role in the pathogenesis of RA in Iraq. The present results agree with those obtained by Nimmisha who demonstrated that the functional R620W variant (rs2476601) in \textit{PTPN22} gene is not present and therefore does not play a role in the pathogenesis of RA in Black South African population\cite{22}.

REFERENCES


