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

Ahmed Yaseen AL-Tarboolii*[1], Hameed Majeed Jassim[2], Mohammed Hadi Alosami[3], [4]Saleh Ahmed Wohaieb

[1]College of Science/Al-Nahrain University
[2]College of Applied Biotechnology/Al-Nahrain University
[3]College of Medicine /Baghdad University
[4]College of Applied Biotechnology/Al-Nahrain University

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***Corresponding Author**
Ahmed Yaseen AL-Tarboolii
College of Science/Al-Nahrain University

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

Primer	Sequence (5'→ 3')	Product Size (bp)
Forward	AAGAATAAGCAAAAACCTCCTGGG	684
Reverse	AACATTGAAAGGACCTGAGAAGT	

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 pateints 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].

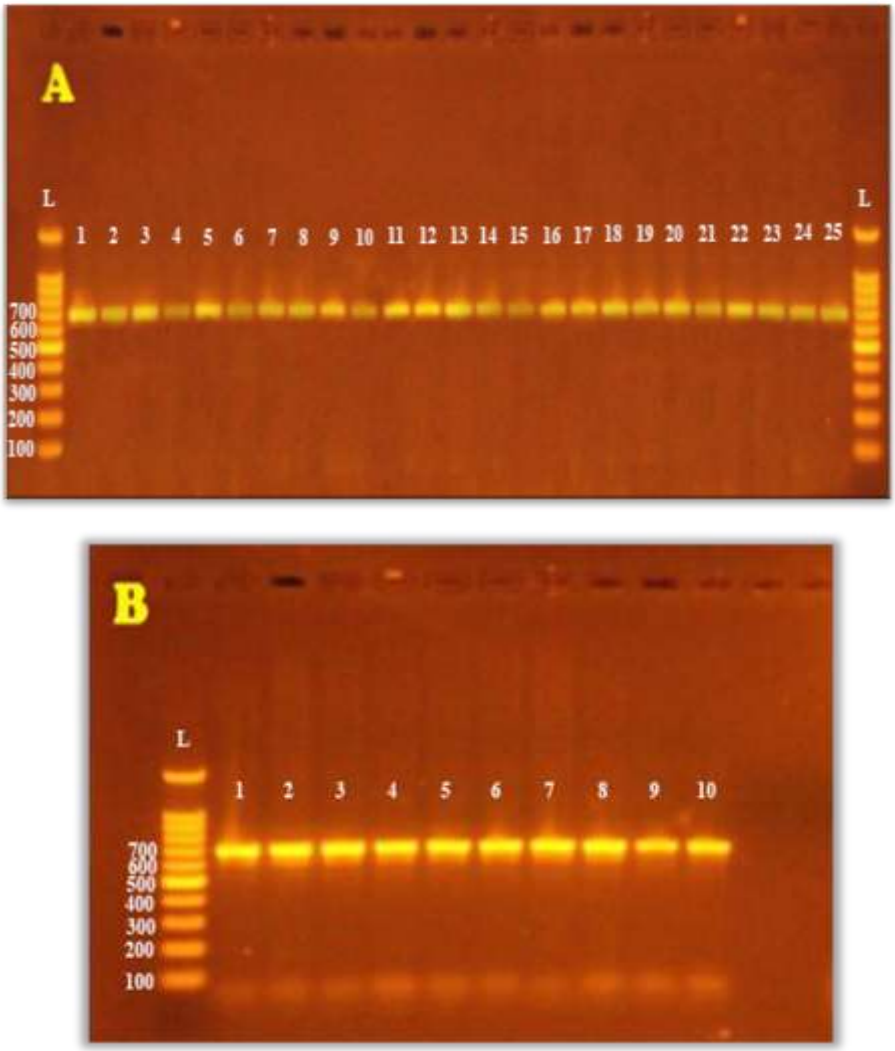

Figure 1: Gel electrophoresis for PCR products of *PTPN22* gene exon 14 on agarose gel (1.8%) after electrophoresis for 1 hour at 5 v/cm^2 in the presence of 1 kb DNA Ladder marker. (A) PCR products for DNA extracted from blood samples of patients with Rheumatoid arthritis. (B) PCR products for DNA extracted form 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|.

Score	Expect	Identities	Gaps	Strand
1118 bits(605)	0.0	605/605(100%)	/605(0%)	Plus/Plus

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Query 1      TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38554  TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 38613

Query 61     TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38614  TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 38673

Query 121    GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38674  GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 38733

Query 181    CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38734  CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 38793

Query 241    AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38794  AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 38853

Query 301    GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38854  GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 38913

Query 361    CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGC 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38914  CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTAATTTAGC 38973

Query 421    AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 38974  AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 39033

Query 481    ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 39034  ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 39093

Query 541    GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 39094  GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 39153

Query 601    TTCTC 605
             |||||
Sbjct39154   TTCTC 39158
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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|

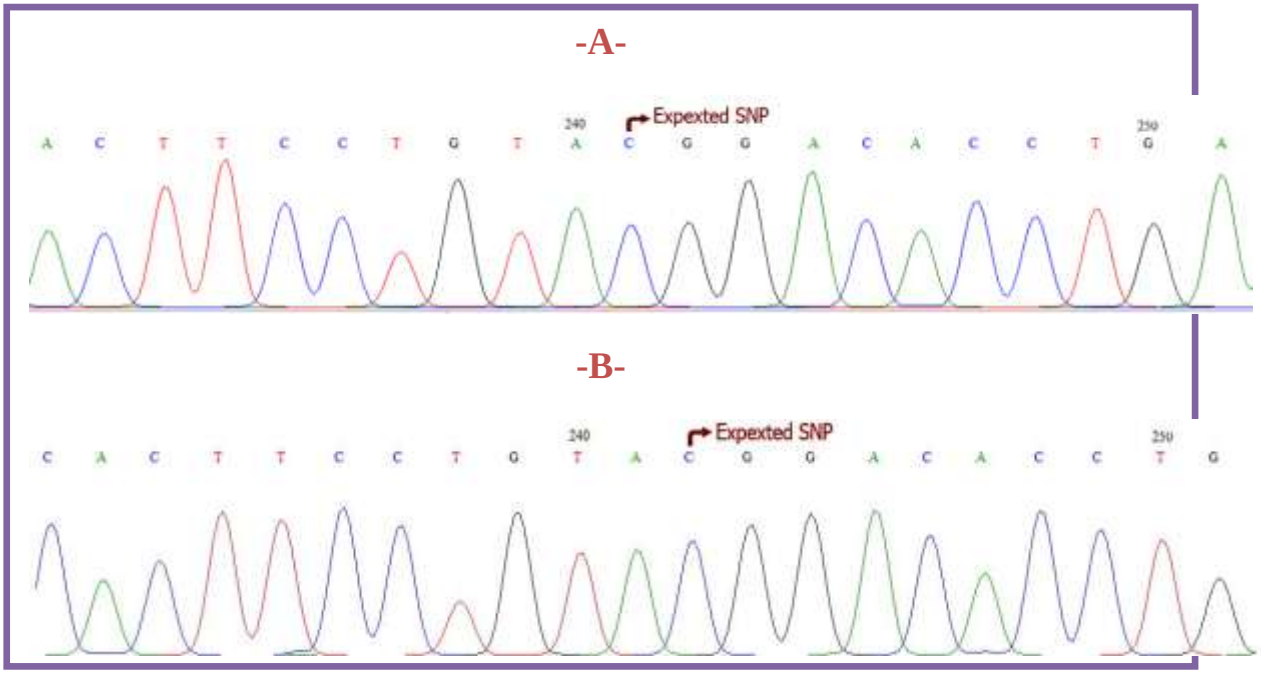
Score	Expect	Identities	Gaps	Strand
1120 bits(606)	0.0	606/606(100%)	0/606(0%)	Plus/Plus

```
Query  1      TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41613  TGAACAAGTGTCAACTTTACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGG 41672

Query  61     TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41673  TAAATTTATATTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATT 41732

Query  121    GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41733  GTTACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAATCCCC 41792

Query  181    CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41793  CCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGAAGCTGGTGAGTAC 41852

Query  241    AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41853  AGTTCAGTAAGTATAAAATAAAGTGTGGGATGGGCATGGTGGCTCATGCCTTTAATTCCA 41912

Query  301    GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41913  GCACTTTGGGAAGCTGATGTGTGAGCCTTGAGTTTGAGGAGTTCATTGAGGCCAGGAGTT 41972

Query  361    CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTTAATTTAGC 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41973  CAAGACTAGCCTGCGCAACATAGTGAGACCTCATCTCTAATTTTTTTTTTTTTAATTTAGC 42032

Query  421   AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42033 AGAGCAATAGCAGCATGCATGTGTAGTCCCAACTATTTGGATGGTGGAGGTGAGAGGATC 42092

Query  481   ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 42093  ACTTGAGCCCAGGAGTTGGGGGCTGCAGTAAGCCATGATTGTGCCACTGCACTCCAGTCT 42152

Query  541   GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42153 GGGTGACAGAGCAAGACCCTGTCTCAAAAAATAAAATGAAGTGTGAATTAACTAGATTAC 42212

Query  601   TTCTCA  606
             ||||||
Sbjct  42213 TTCTCA  42218
```

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; Sbject 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.

Number of samples	Accession	Compatibility	Expect	Score	Number of nucleotides
10 Controls	gb|EF064714.1|	100 %	0.0	1118	38554 - 39158
25 Patients	ref|NG_011432.1|	100 %	0.0	1120	41613-42218



In total, 35 samples (25 RA patients and 10 healthy controls) were analyzed for the rs2476601 of *PTPN22* gene. Results of analyzed sequence shower 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.

Samples	N	Genotypes			Alleles (Frequency)	
		C/C	C/T	T/T	C	T
Controls	10	10	0	0	1.00	0.00
RA Patients	25	25	0	0	1.00	0.00
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 *PTPN22*, other than rs247660 do not play a role in the incidence of RA in Iraqi population. In the Japanese, *PTPN22* allelic heterogeneity was studied, but none of the 8 other SNPs present in the European populations, were found to be polymorphic[18].

Another explanation may be that the variants in the *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[21].

In conclusion, the presence of imbalance of cytokine production oxidative stress in the absence of the *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 *PTPN22*geneis not present and therefore does not play a role in the pathogenesis of RA in Black South African population [22].

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