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GENETIC POLYMORPHISM IN ENDOPLASMIC RETICULUM AMINOPEPTIDASE-L (*ERAP1*) GENE IN IRAQI PATIENTS WITH ANKYLOSING SPONDYLITIS

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ABSTRACT

Ankylosing spondylitis (AS) is a common, highly heritable inflammatory arthritis affecting primarily the spine and pelvis. This study was aimed to investigate the relationship between the genetic polymorphism in *Endoplasmic reticulum aminopeptidase-1 (ERAP1) gene* in the position 96144608 for rs27044 SNP in exon15 within chromosome 5 with the susceptibility and severity of the disease in a sample of Iraqi patients, as this SNP was regarded as a major risk factor associated with incidence of AS . A total of 35 blood samples (25 AS patients and 10 healthy controls) were recruited in this study. Genomic DNA was extracted from those blood samples, and it was found that the concentration of extracted DNA was ranged between 200-400 pg/ml with purity of 1.8-2.0. Exon 15 of *ERAP1* was amplified by using specific primers designed in this study. Results of amplification showed that a single DNA fragment of 298 bp was obtained after electrophoresis on 2% agarose gel represents the complete nucleotide sequence of exon 15. To investigate the genetic polymorphism in this exon, the nucleotide sequence for the amplified fragment was determined. Results of sequencing showed that the single nucleotide polymorphism in position 96144608 for the rs27044 was cSNP in all healthy controls group, while gSNP was found in 64% polymorphic AS patients with a significant difference than non-polymorphic AS patients (36%), which refers that this variation was regarded as a risk factor associated with the susceptibility of AS in a sample of Iraqi population.

KEYWORDS: *Endoplasmic reticulum aminopeptidase-1 (ERAP1) gene.*

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic and systemic seronegative inflammatory spondyloarthropathy, which causes destruction and fusion of the spinal vertebrae and sacroiliac joints.[1] It has been proposed that the sites of attachment of the ligaments or tendons to the bone, called entheses, are the major target of the inflammatory, traumatic and degenerative pathological changes occurring in AS.[2] Enthesitis is believed to play a primary role in the ligament calcification process, which results in pain. It can lead to reduced flexibility of the spine, and eventually complete loss of spinal mobility, destruction as well as ankylosis (fusion) of the spine and sacroiliac joints.[3]

Recently, genomic studies of patients with AS have identified and validated other loci aside from the HLA-B27 that are involved in the pathogenesis of this disease. These genes include the *endoplasmic reticulum- associated aminopeptidase 1 (ERAP1), IL-23 receptor (IL-23R), IL-1 receptor (IL-1RII),* and two loci that code for unknown genes.[4]

ERAP1 is a zinc aminopeptidase belonging to the Ml family of the metallopeptidases with several proposed biological functions that make it a strong candidate in AS.[5] The association of single nucleotide polymorphisms (SNPs) in *ERAP1* with AS can be explained from a functional perspective. The protein *ERAP1* has three known biological functions. First, in the endoplasmic reticulum, *ERAP1* acts as a molecular ruler, trimming peptide antigens to optimal length for binding to MHC class I molecules.[6] Second, the cleavage of cell surface receptors by proinflammatory cytokines results in the down-regulation of their intracellular signaling.[7] Third, *ERAP1* is involved in the activation of macrophages induced by lipopolysaccharide (LPS) and interferon (IFN)-y.[8]

In 2007, the Welcome Trust Case Control Consortium and the Australo-Anglo-American Spondylitis Consortium (WTCCC-TASC) performed a genetargeted association study of 14500 nsSNPs in 1000 AS cases and 1500 controls. They confirmed the known strong association with the major histocompatibilty complex (MHC) and also provided preliminary evidence for several non-MHC associations. Five nsSNPs in *ERAP1* (rs30187, rs27044, rs2887987, rs17482078 and rs10050860) were genotyped, all producing significant associations with AS.[9] According to those findings, this study was aimed to determine the relationship between the genetic polymorphism and in *ERAP1* and the incidence of Ankylosing spondylitis in a sample of Iraqi patients.

MATERIALS AND METHODS

Subjects

Blood samples (3-5 ml) were collected in EDTA anticoagulant tubes from 25 male and female Ankylosing spondylitis patients (either newly diagnosed or already treated patients) 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 *Endoplasmic Reticulum Aminopeptidase 1* Exon 15

Amplification of *ERAP1* exon 15 was carried out by using specific primers indicated in table (1). These primers were designed in this study and were supplied by Alpha DNA Company. A working solution of 10 pmol/µl of these primers was prepared by adding 10 µl of primer stock solution to 90 µl of deionized distilled water to get a final concentration of 10 pmol/µl.

Table (1): Oligonucleotide primers used for the amplification of *ERAP1* exon 15

Primers	Sequence (5'- 3')	Product Size (bp)
Forward	CAGGGGAGACACTTAAACTT	298
Reverse	ACTACTCCTTCCGGACTC	

PCR amplification program for Exon 15 of *ERAP1* was achieved by initial denaturation for one cycle at 94 °C for 1min., denaturation and annealing for forty cycles at 94 °C for 30 sec., then extension for forty cycles for 1min. at 72 °C and final extension for one cycle for 1min. at 72 °C

Sequencing of amplification products

Amplification products for exon 15 of *ERAP1* were sequenced 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 *ERAP1* gene, using (Bioedit) software.

RESULTS AND DISCUSSION

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.[10] Polymerase chain reaction was used to amplify exon 15 of *ERAP1* in the extracted genomic DNA under optimum amplification conditions by using specific primers shown previously. Results illustrated in figure (1) showed that the amplified products were appeared as clear bands after electrophoresis on agarose gel (2%) with a molecular size of 298 base pair for each blood sample obtained from healthy controls, newly diagnosed AS patients and medically treated patients. These fragments represent the amplified fragment of exon 15 of *ERAP1* gene located on long arm of chromosome 5. The real molecular size of this exon is 184 bp in length, while the molecular size of the amplified fragment was 298 bp and that's because both reverse and forward primers were designed to amplify the completed sequence of exon 15 in addition to the two border sequences located on either sides of the exon.

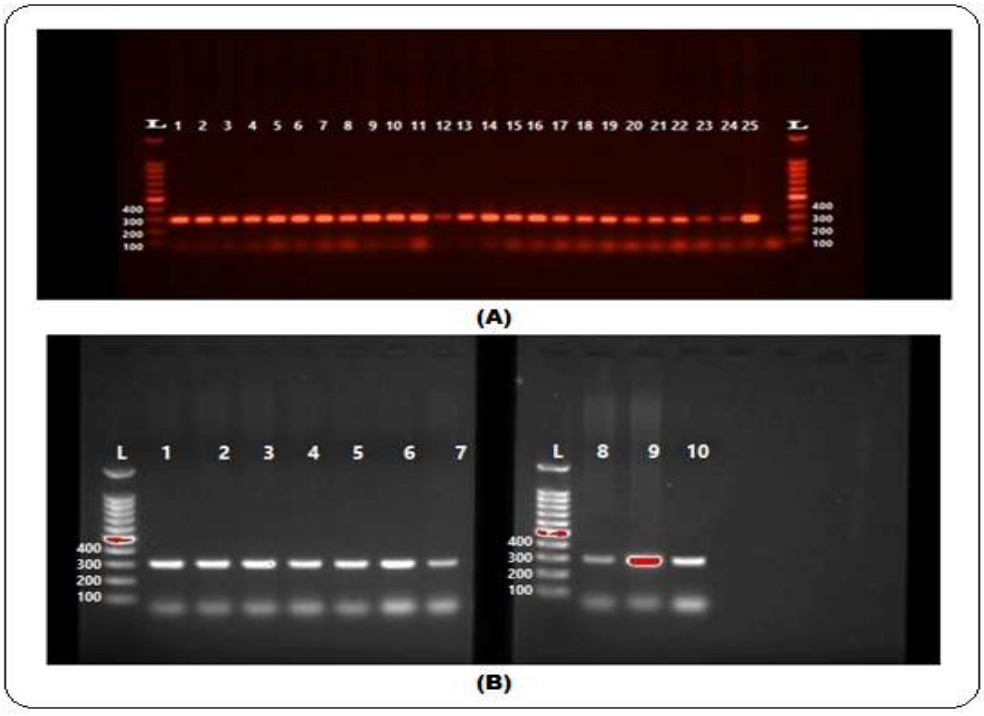

Figure (1): Gel electrophoresis for PCR products of *ERAP1* exon 15 on agarose gel (2%) after electrophoresis for 1 hour at 5v/cm^2 volt in the presence of 1 kb DNA Ladder marker. (A): PCR products for amplified DNA extracted from blood samples of patients with Ankylosing spondylitis. (B): PCR products for amplified DNA extracted form blood samples of healthy controls

Sequencing of Amplified Exon 15 of *ERAP1* Gene

Endoplasmic reticulum aminopeptidase1 (*ERAP1*) gene is one of the important non-HLA genes associated with susceptibility of Ankylosing spondylitis. Single nucleotide polymorphisms (SNPs) in *ERAP1* gene, such as rs27044, are highly associated with the disease in several ethnic populations.[11] Several studies mentioned that the most effective genetic risk outside of the HLA region is a functional single nucleotide polymorphism in the *ERAP1* gene which is a C→G substitution (rs27044), which results in a substitution of Glutamine (Q) for Glutamic acid (E) at codon 671.[12]

Exon 15 was selected to study the role of the *ERAP1* SNP (rs27044) polymorphism in disease susceptibility and severity in twenty five Iraqi AS patients to investigate the relationship between rs27044 SNP in exon 15 of *ERAP1* gene and the severity of the disease compared with ten healthy controls. The complete nucleotide sequence of *ERAP1*, exon 15 was determined for genotyping the genetic polymorphism in this exon. Results illustrated in figure (2) showed the complete nucleotide sequence of this exon in healthy controls and the position of the expected rs27044 SNP that may be associated with the incidence of Ankylosing spondylitis. This sequence was identical to the nucleotide sequence of this exon for the reference sequence of *ERAP1* recorded in (NCBI Reference Sequence: *ERAP1* ID: 51752).

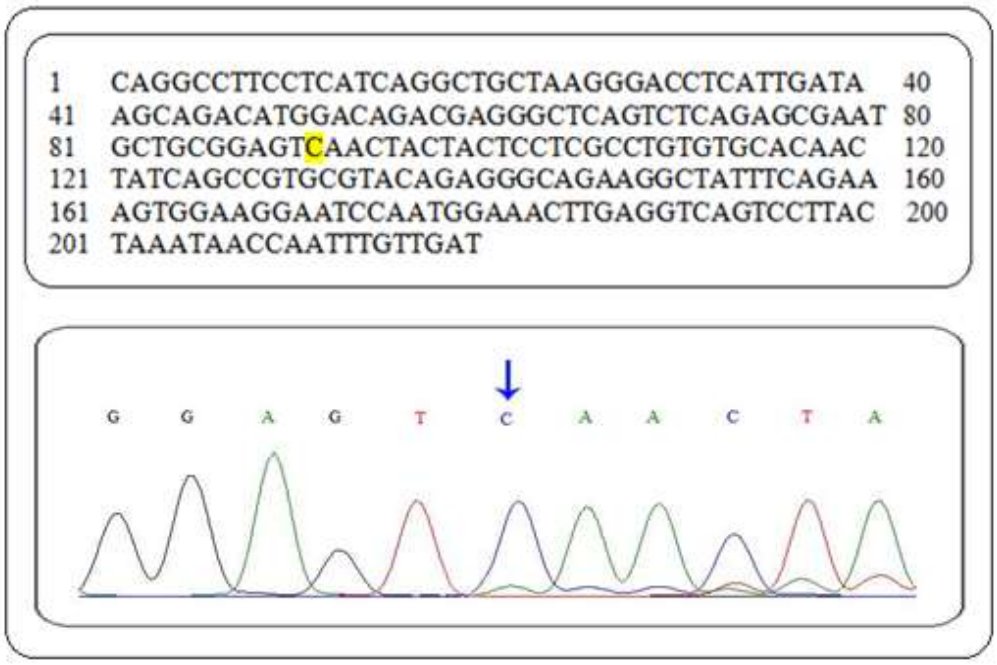

Figure (2): Nucleotide sequence of exon 15 of *ERAP1* in healthy controls group. Yellow letter indicates the position (96144608) of expected SNP in a sample of Iraqi population

Nucleotide Sequence Alignment of the Amplified Exon 15 of *ERAP1*

Results of the sequence analysis was analyzed by blast in the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect rs27044 polymorphism in exon 15 of *ERAP1* gene. Results illustrated in figure (3) and table (2) showed the nucleotide 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: ref|NG_027839.1| from 35907-36126 number of nucleotide from *Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1).*

Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1), RefSeqGene on chromosome 5, Sequence ID: ref|NG_027839.1|

Score	Expect	Identities	Gaps	Strand
407 bits(220)	5e-110	220/220(100%)	0/220(0%)	Plus/Plus

```
Query  1      CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATAAGCAGACATGGACAGACGAG 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35907  CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATAAGCAGACATGGACAGACGAG 35966

Query  61     GGCTCAGTCTCAGAGCGAATGCTGCGGAGTCAACTACTACTCCTCGCCTGTGTGCACAAC 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35967  GGCTCAGTCTCAGAGCGAATGCTGCGGAGTCAACTACTACTCCTCGCCTGTGTGCACAAC 36026

Query  121    TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAAAGTGGAAGGAATCCAATGGA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36027  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAAAGTGGAAGGAATCCAATGGA 36086

Query  181    AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  220
              ||||||||||||||||||||||||||||||||||||||||
Sbjct  36087  AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  36126
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Figure (3): Alignment between the nucleotide sequence of healthy control samples of Iraqi and reference sequence. Query represents healthy control samples; Sbject represent of database of National Center for Biotechnology Information (NCBI). Yellow letters indicates the position of matched SNP

On the other hand, results of the nucleotide sequence for *ERAP1* exon 15 in Iraqi patient with Ankylosing spondylitis indicated in table (2) showed that 9 (36%) out of 25 patients were genotyped as homozygous CC and non-polymorphic as compared to the healthy controls because the expected SNP (rs27044) does not occur in exon 15 sequence understudy, hence the Cytosine nucleotide was located in the same position as in the healthy controls without any base substitution to other nucleotides compared to reference sequence. These

results are also observed in other ethnic populations such as some Taiwanese population which were non-polymorphic in their nucleotide sequence of this exon.[12]

Table (2): Genotyping and alleles frequency in Ankylosing spondylitis patients and healthy controls

Sample	No.	Genotypes			Allele frequency	
		CC (%)	GC (%)	GG (%)	C	G
Controls	10	**10** (100.00)	**0** (0.00)	**0** (0.00)	1.00	0.00
AS Patients	25	**9** (36.00)	**16** (64.00)	**0** (0.00)	0.68	0.32

Results indicated in table (3) showed the C nucleotide (cSNP) of non-polymorphic Ankylosing spondylitis patients was identical to C nucleotide in reference sequence mentioned in NCBI, with 100% identity, under sequence ID: ref|NG_027839.1| from 35907-36126 number of nucleotide from *Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1).*

On the other hand, results illustrated in figure (4) and table (3) also showed that 16(64%) of Iraqi patients with Ankylosing spondylitis were genotyped as heterozygous GC and were positive for rs27044 SNP exon 15 of *ERAP1*, as cytosine nucleotide was substituted by guanine.

Table (3): Sequencing ID in gene bank, identity, expect, score and range of DNA sequence obtained from National Center Biotechnology Information (NCBI)

Sample	No.	SNP	Identity (%)	Expect	Score	Range
Healthy controls	10	C nucleotide	100	5e-110	407	35907 - 36126
AS patients	9	C nucleotide	100	5e-110	407	35907 - 36126
	16	G nucleotide	99	2e-108	401	35907 - 36126

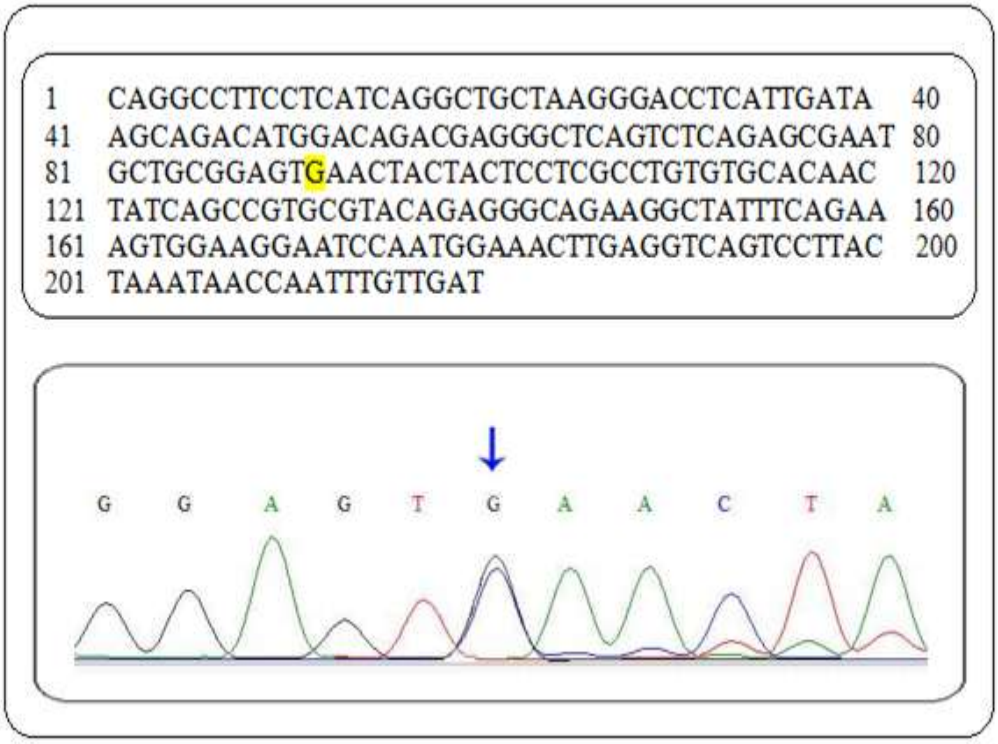

Figure (4): Nucleotide sequence of exon 15 of *ERAP1* in Ankylosing spondylitis patients group. Yellow letter indicates the position of SNP in Iraqi population

Furthermore, results indicated in table (3) showed the position of the G nucleotide (gSNP) of polymorphic Ankylosing spondylitis patients matched with C nucleotide in reference sequence mentioned in NCBI, with 99% identity, under sequence ID: ref|NG_027839.1| from 35907-36126 number of nucleotide from *Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1).*

Alignment of Amino Acids

The amino acids sequence encoded by exon 15 in *ERAP1* gene, in healthy controls and Iraqi patients with Ankylosing spondylitis were examined and compared with the reference sequence recorded in NCBI. Results illustrated in figure (5-a) showed the amino acids alignment between healthy controls and reference sequence, under sequence ID: pdb|3RJO|A, and the position of amino acid (Glutamine, Q) related to the expected rs27044 SNP that may be occurred in this exon. These results showed that there is no any change in the sequence of amino acids (100% identity) between healthy controls and reference sequence. On the other hand, results illustrated in figure (5-b) showed that the amino acid (Glutamine, Q) related to the expect rs27044 SNP was the same in non-polymorphic Iraqi patients with ankylosing

spondylitis and reference sequence (100% identity) because those patients does not suffering from any polymorphism.

Amino acid alignment between Iraqi patients with Ankylosing spondylitis (gSNP) and reference *ERAP1*, under sequence ID: pdb|3RJO|A**,** was shown in figure (5-c). As a consequence of the substitution of Cytosine by Guanine in exon 15 of *ERAP1*gene in those patients, the amino acid sequence result from the wild-type allele was changed, and the amino acid encoded by the original codon was changed to another codon encoding to different amino acid as Glutamine (Q) was substituted by Glutamic acid (E). occurring of transversion mutation in this position causing missense mutation leading to change neutral, polar amino acid (Gln) to negatively charged, polar aliphatic amino acid (Glu) as indicated in table (4) may alter the structure and function of *ERAP1* enzyme may associated with the incidence of Ankylosing spondylitis as the malfunctioning of *ERAP1* would lead to either an increase or decrease in the number of cell surface receptors available for cytokine (IL-6 and IL-23), thus propitiating pro-inflammatory effects and finally raising disease susceptibility to Ankylosing spondylitis.[11]

This alteration in ERAP1 structure may cause the evaluation of Interlukin-6 and Interlukin-23 cytokines levels[7], and immunological parameters levels (C-reactive protein and HLA-B27) as well as the change in oxidative stress parameters levels (Malondialdehyde and Glutathione). Furthermore this SNP was a risk factor for the susceptibility to the disease because this change revealed a highly response for the infection with this disease.[13]

These results agree with those obtained by Szczypiorska *et al*, who mentioned that cSNP substituted with gSNP in a sample of Spanish population.[13] These results also agree with those obtained by Choi *et al*, and Wang *et al*, who mentioned that cSNP substituted with gSNP in a sample of Korean and Taiwanese population respectively.[14, 15]

Figure (5): Alignment of amino acid encoded by exon 15 of *ERAP1* gene in Ankylosing spondylitis Iraqi patients (Query) and with the reference sequence (Sbject) scored by National Center Biotechnology Information (NCBI). Yellow letters indicates the position of the expected polymorphic amino acid. (A): Healthy controls. (B): Non-polymorphic patients. (C): Polymorphic patients

Table (4): Type of polymorphism in *Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1)*

Type of substitution	Predicted effect	Amino acid change	No. of patient (%)	Nucleotide change	Location of Cod in gene bank	Location of polymorphic nucleotide
Transversion	Missense	Glutamine (Q) / Glutamic acid (E)	16 (64%)	CAA> GAA	201	35996

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