Genetic Polymorphism for the Gene *Encoding Endoplasmic Reticulum Aminopeptidase-1 (ERAP-1)* 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 rs27044 polymorphism in *Endoplasmic reticulum aminopeptidase-1 (ERAP-1)* with the susceptibility and severity of AS correlated with some biochemical markers such as hematological parameter (Erythrocytes sedimentation rate (ESR)) and immunological parameters (C-reactive protein (CRP), Human leukocyte antigen-B27 (HLA-B27), Interlukin-6 (IL-6) and Interlukin-23 (IL-23)), and oxidative stress parameters (Glutathione (GSH) and Malondialdehyde (MDA)) in a sample of Iraqi population. A total of 60 blood samples were collected from AS patients requited Rheumatology Consultation Clinic in Baghdad Teaching Hospital in Baghdad Governorate. Those patients are males and females with age ranged between 26- 32 years for early diagnosed patients (10 samples) and other medically treated patients with age ranged between 22-52 years (50 samples). The rest (30 blood samples) were collected from healthy controls with age ranged between 16-68 years. Results showed that there is a significant increase in Erythrocytes Sedimentation Rate (ESR) as a hematological parameter in newly diagnosed and medically treated patients. For the immunological parameters, results showed that C-reactive protein (CRP) was detected in all serum samples taken from newly diagnosed patients, and 80% positive were detected in medically treated patients in comparison with 100% negative results in healthy controls. On the other hand, Human leukocyte antigen-B27 (HLA-B27) was also detected in all serum samples taken from newly diagnosed patients and 76% positive were recorded in medically treated patients as compared with negatively resulted in healthy controls. For the Interleukins (IL-6 and IL-23), results showed that there is a significant increase in levels of both interleukins in serum samples of newly diagnosed patients compared with healthy controls and medically treated patients, in which any significant difference was recorded. Results of oxidative stress parameters showed that there is a significant decrease in Glutathione (GSH) levels in serum samples of newly diagnosed patients compared with its level in serum sample of controls and medically treated patients, in which significantly not different. Results also showed that there is a significant increase in Malondialdehyde (MDA) levels in serum samples of newly diagnosed patients compared with its levels in healthy controls and medically treated patients, in which no significant difference in MDA levels in serum samples of both groups. Genetic polymorphism of *ERAP-1* gene (rs27044 SNP) in exon15 within chromosome 5 was studied. The genomic DNA was extracted from blood samples for controls and AS patients at concentrations of extracted DNA was ranged between 100-200 ng/µl with purity of 1.8-2.0. Exon 15 was amplified by using specific primers results showed that a single band of 298 bp was obtained after electrophoresis on agarose gel (2%) represents the complete nucleotide sequence of exon 15. Results showed that the single nucleotide polymorphism (rs27044) was cSNP in all controls, while gSNP was found in 64% of polymorphic AS patients with a significant difference than non-polymorphic AS patients (36%).

Keywords: *Endoplasmic reticulum aminopeptidase-1 (ERAP1) gene, ankylosing spondylitis.*

1. Introduction

Ankylosing spondylitis (AS) is a chronic and systemic seronegative inflammatory spondyloarthropathy, which causes destruction and fusion of the spinal vertebrae and sacroiliac joints. 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. 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 (Ghasemi-rad *et al.,* 2015).

Serum and plasma biomarkers have been examined extensively in assessing disease activity, treatment response, and as predictors or radiographic severity. For assessing disease activity, other than C-reactive protein and erythrocyte sedimentation rate, the most work has been in examining cytokines (particularly interleukin 17 and 23), matrix metalloproteinase (MMP) markers (particularly MMP3). For assessing those at the highest risk for radiographic progression, biomarkers of bony metabolism, cartilage and connective tissue degradation products, and adipokines have been most extensively assessed (Reveille J. D., 2015).

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 (*ERAP-1*), IL-23 receptor (IL-23R), IL-1 receptor (IL-1RII), and two loci that code for unknown genes. The risk attributed to populations with the three known associated genes is 90%, 26%, and 1%, respectively (Reveille *et al.,* 2010).

Endoplasmic Reticulum Aminopeptidase-1 (ERAP-1) is the term currently accepted by the human genome organization (HUGO) nomenclature committee (HGNC), though in the past it was known by such names as endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), adipocyte-derived leucine amino peptidase (A-LAP), and aminopeptidase regulating tumor necrosis factor receptor I (TNFRI) shedding (ARTS-1). *ERAP-1* is a zinc aminopeptidase belonging to the M1 family of the metallopeptidases with several proposed biological functions that make it a strong candidate in AS (Kochan *et al.,* 2011).

The association of *ERAP-1* SNPs with AS can be explained from a functional perspective. The protein *ERAP-1* has three known biological functions. First, in the endoplasmic reticulum, *ERAP-1* acts as a molecular ruler, trimming peptide antigens to optimal length for binding to MHC class I molecules (Saveanu *et al.,* 2005). Second, the cleavage of cell surface receptors by pro-inflammatory cytokines results in the down-regulation of their intracellular signaling (Cui *et al.,* 2003). Third, *ERAP-1* is involved in the activation of macrophages induced by lipopolysaccharide (LPS) and interferon (IFN)-γ (Goto *et al.,* 2011).

In 2007, the Wellcome 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 *ERAP-1* (rs30187, rs27044, rs2887987, rs17482078 and rs10050860) were genotyped, all producing significant associations with AS (Burton *et al.,* 2007).

According to those mentioned above and to determine the relationship between the genetic polymorphism in *ERAP-1* gene and the incidence of ankylosing spondylitis in a sample of Iraqi population, this study was amid to collection of blood samples from patients diagnosed with ankylosing spondylitis, measurement of some inflammatory markers and immunological markers,

measurement of some biochemical parameters and markers of oxidative stress, extraction the genomic DNA from Iraqi healthy controls and AS patients, amplification of exon 15 of *ERAP-1* by designing a specific primer and then, sequencing the amplified PCR products and determine the presence of genetic polymorphism in this exon.

2. Materials and methods

2.1. SUBJECTS

Blood samples were collected from 60 male and female Ankylosing spondylitis patients (with either newly diagnosed or already treated patients) from Rheumatology consultation clinic in Baghdad Teaching Hospital. The Ankylosing spondylitis patients included 10 newly diagnosed (untreated) patients (8 men and 2 women) with an age range of 26-32 year. Also, another 50 already diagnosed as having Ankylosing spondylitis and currently receiving treatment included 44 men and 6 women with an age range of 22 – 54 year. The range of diseases duration was between three months to 30 yr. Before blood sampling, all participants were informed about the study objectives, and their consent were obtained. Another blood samples were collected from 30 apparently healthy controls from Al-Harthia laboratory. They included 20 women and 10 men with an age range of 16 – 68 year.

Medically treated patients were received several types of drugs for curing the disease, such as Methotrexate (MTX), Humera, Prednisolone, Biology, Immuron, Hydroxychloroquine, Enbrel and Salazopyrin. Other patients suffering from Ankylosing spondylitis were not received any medical treatment.

2.2. DETERMINATION OF C-REACTIVE PROTEIN

C-reactive protein (CRP) was determined in serum sample at 6 mg/l or higher causes a visible agglutination on slide of a suspension of latex particles coated with anti-human C-reactive protein.

2.3. HUMAN LEUKOCYTE ANTIGEN B27 (HLA-B27) ELISA KIT

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to HLA-B27. Standards or samples were added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for HLA-B27 and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain HLA-B27, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of HLA-B27. One can calculate the concentration of HLA-B27 in the samples by comparing the OD of the samples to the standard curve.

2.4. DETECTION OF INTERLEUKIN-6 (IL-6)

Interleukin 6 (IL-6) was determined in serum samples by using IL-6 ELIAS Kit. This kit uses enzyme- linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Interleukin 6. Interleukin 6 was added to wells that are pre-coated with Interleukin 6 monoclonal antibody and then incubate. After incubation, anti-Interleukin 6 antibodies labeled with biotin were added to unite with Streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washing, and then substrate A and B were added. The solution will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of Human Interleukin 6 are positively correlated.

2.5. DETECTION OF INTERLEUKIN-23 (IL-23)

Interleukin 23 (IL-23) in serum samples was determined by using IL-23 ELIAS Kit. This kit uses enzyme- linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Interleukin 23. Interleukin 23 was added to wells that are pre-

coated with Interleukin 23 monoclonal antibody and then incubated. After incubation, anti-Interleukin 23 antibodies labeled with biotin were added to unite with Streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washing, and then substrate A and B were added. The solution will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of Human Interleukin 23 are positively correlated.

2.6. DETECTION OF GLUTATHIONE (GSH)

Glutathione was detected in blood serum from patients and healthy controls by using GSH ELISA Kit. This kit uses enzyme- linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human glutathione (GSH). Glutathione (GSH) was added to wells that are pre-coated with glutathione (GSH) monoclonal antibody and then incubated. After incubation, anti-glutathione (GSH) antibodies labeled with biotin were added to unite with Streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washing, then substrate A and B was added. The solution will turn blue and change to yellow with the effect of acid. The shades of solution and the concentration of Human glutathione (GSH) are positively correlated.

2.7. DETECTION OF MALONDIALDEHYDE (BUEGE AND AUST, 1978)

1. HCl Solution (0.25 N): This solution was prepared by adding 2.08 ml of concentrated hydrochloric acid to 98 ml of distilled water.

2. Thiobarbituric Acid (TBA) Reagent: This reagent was prepared by dissolving 0.375 g of Thiobarbituric acid (TBA) and 15 g of trichloroacetic acid (TCA) in 100 ml of 0.25 N of hydrochloric acid solution.

3. Malondialdehyde assay procedure: Malondialdehyde (MDA) concentration in serum samples were assayed as follows:

A. Aliquot of 0.5 ml of serum samples were added to 1 ml of TBA reagent and mixed thoroughly, then mixture was incubated at 100°C for 15 minutes.

B. The mixture was centrifuge at 14000 rpm for 10 minutes.

C. After centrifugation, optical density for supernatant was measured at 532 nm against blank of distilled water (0.5 ml) and TBA reagent (1 ml).

D. Malondialdehyde concentration was calculated according to the following formula (Buege and Aust, 1978).

2.8. DNA EXTRACTION

Total genomic DNA was extracted from 25 blood samples collected from Ankylosing spondylitis patients, and from 10 blood samples collected from healthy volunteers. DNA extraction was carried out by using ReliaPrep™ Blood gDNA Miniprep System supplied by Promega / USA protocol.

2.9. PCR PRIMERS

Amplification of *endoplasmic reticulum aminopeptidase-1* (*ERAP-1*) was achieved by using specific primers indicated in Table-1. These primers 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 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 *ERAP-1*

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

2.10. AMPLIFICATION OF *ERAP-1* EXON 15

PCR was carried out in a total volume of 50ul. The reaction components are indicated in Table-2. Exon 15 of *ERAP-1*gene was amplified by using specific primers in a thermal cycler. Polymerase chain reaction was carried out according to the amplification program shown in Table-3, and then amplification products were analyzed on agarose gel (2%) in presence of 100pb DNA ladder marker.

Table-2: Components of reaction mixture for amplification of exon 15 of *ERAP-1*

Component	Volume (µl)
Master Mix: TaqDNA Polymerase, dNTPs, MgCl2, and reaction buffer.	25
Forward Primer	2.5
Reverse Primer	2.5
DNA Template	7.5
D.W.	12.5

Table-3: PCR amplification program for Exon 15 of *ERAP-1*

Steps	Temperature (℃)	Time	No. of Cycles
Initial Denaturation	94	5 min.	1
Denaturation	94	30 sec.	
Annealing	58	30 sec.	40
Extension	72	1 min	
Final Extension	72	10 min.	1

2.11. SEQUENCING OF AMPLIFICATION PRODUCTS

Amplification products for exon 15 of the gene *ERAP-1* 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 *ERAP-1* gene, using (Mega-6) software.

2.12. STATISTICAL ANALYSIS

The Statistical Analysis System- SAS (2012) program was used to evaluate difference factors in study parameters. Chi-square test was used to investigate significant comparisons between percentages and the least significant difference –LSD test was used to significant compare between means of the present parameter. Estimation of correlation coefficient between some parameters was performed, as well.

3. Results and Discussion

3.1 LEVEL OF ERYTHROCYTE SEDIMENTATION RATE

Erythrocyte sedimentation rate (ESR) is the most widely used laboratory measure of disease activity in clinical medicine and still remains a useful tool for monitoring inflammatory diseases such as Ankylosing spondylitis (Wolfe *et al.,* 2001). Also it was the most frequently indicator used for detection the inflammatory activity in patients with Ankylosing spondylitis (Wallis *et al.,* 2013).

In this study, ESR was detected in whole blood samples of 60 patients with Ankylosing spondylitis and other 30 healthy control persons. Results indicated in table (3-1) showed that, the level of ESR was significantly increased in medically treated and newly diagnosed Ankylosing spondylitis patients in comparison with its levels in healthy controls (P = 0.0012). The increase in ESR levels in newly diagnosed and treated Ankylosing spondylitis patients refers to the presence of an inflammatory response by the immune system against the disease. This finding agrees with other studies described by Dixon *et al* (1981); Benjamin *et al* (2001); Londono *et al* (2012) who demonstrated higher ESR levels in blood samples of AS patients compared to healthy controls. The failure of various treatments employed to

normalize ESR values may be due to the presence of other mechanisms responsible for the

pathogenesis of AS not corrected by the current therapy.

Table (3-1): Erythrocyte sedimentation rate in healthy control and Ankylosing spondylitis Patients

Parameters	Controls N=30	AS Patients	
		Newly diagnosed N=10	Treated N= 50
ESR (mm/hr)	21.07 ± 3.79[b]	58.40±3.04[a]	49.77 ± 11.42[a]

Values are expressed as Mean ± SD. N=Number, different letters indicate
a significant difference at P<0.01.

3.2. IMMUNOLOGICAL PARAMETERS

3.2.1. C-reactive protein

C-reactive protein (CRP) and ESR are two currently used biomarkers for the evaluation of inflammatory activity of Ankylosing spondylitis (Spoorenberg *et al.,* 1999).

In this study, Results indicated in table (3-2) showed that C-reactive protein was detected in serum samples of all ten newly diagnosed patients with Ankylosing spondylitis (100.0%), and in the forty patients (80%) among the fifty treated patients. While this protein was not detected in serum samples of all healthy controls (0.00%) with a highly

significant difference (P= 0.0001). These results showed that the CRP test is one of the most important immunological biomarkers for the diagnosis of AS, and was highly related to the infection with the disease.

These results are agreed with those obtained by Londono *et al* (2012) who mentioned that higher CRP levels was present in serum of AS patients compared with healthy controls, and also agreed with Przepiera-Bwdzak *et al* (2015) who detect high levels of CRP in all patients with AS in comparison with the healthy controls (0.0%).

Table (3-2): levels of C-reactive protein in Iraqi patients with Ankylosing spondylitis and healthy controls

Parameters	Controls N (%)	AS patients	
		Newly diagnosed N (%)	Treated N (%)
CRP			
Positive	0 (0.00)	10 (100.0)	40 (80.0)
Negative	30 (100.0)	0 (0.00)	10 (20.0)

Values are expressed as Mean ± SD. N=Number, (%) = Percentage, ** (P<0.01).

3.2.2. Human Leukocyte Antigen B27

Typing for histocompatibility antigen HLA-B27 has been suggested as a clinically valuable diagnostic test for Ankylosing spondylitis (Londoño *et al.,* 2005). A genetic predisposition exists for the development of the disease, as evidenced by its strong association with HLA-B27, especially in the case of AS, where 90% of the patients are positive for this allele (Khan *et al.,* 1992).

In this study, Results indicated in table (3-3) showed that Human Leukocyte Antigen B27 was detected in serum samples of all ten newly diagnosed patients with Ankylosing spondylitis (100.0%) with a highly significant difference (P=0.0001) as compared to the healthy controls, and in serum samples of thirty eight treated patients (76.0%), among 50 treated patients, while this protein was not detected in serum samples of all healthy

controls (0.00%) with a highly significant difference (P<0.01). According to these results, the HLA-B27 test was regarded as one of the most important immunological biomarkers in the diagnosis of AS, and was highly related to the infection with the disease.

These findings were agreed with that obtained by Chen *et al* (2012) who found that the positivity of HLA-B27 test in patients with AS was 99.2%.

Table (3-3): levels of Human Leukocyte Antigen B27 in Iraqi patients with Ankylosing spondylitis and healthy controls

Parameters	Controls N (%)	AS patients	
		Newly diagnosed N (%)	Treated N (%)
HLA-B27			
Positive	0 (0.00)	10 (100.0)	38(76.0)
Negative	30 (100.0)	0 (0.00)	12 (24.0)

Values are expressed as Mean ± SD. N=Number, (%) = Percentage, ** (P<0.01).

3.2.3. Interleukin-6

Some cytokines such as interleukin-6 (IL-6) and interleukin-23 (IL-23) are considered to be associated with the pathogenesis of chronic inflammatory joint disease (Cauli and Mathieu, 2012). IL-6 is a pleiotropic cytokine that plays role in arthritis but its role in the pathogenesis of AS remains controversial (Hayer *et al.,* 2010).

Levels of IL-6 in Iraqi patients with AS and healthy controls was determined. Results indicated in figure (3-1) showed that the mean level of IL-6 in newly diagnosed and medically treated patients with AS was 258.23 ng/ml and 68.97 ng/ml respectively in comparison with 61.47 ng/ml in healthy controls. On the other hand, results illustrated in figure (3-1) showed that there is a significant increase in serum levels of IL-6 in newly diagnosed Ankylosing spondylitis patients compared with healthy controls (P<0.01). Furthermore, medical treatment of those patients showed no any significantly difference in IL-6 levels at a value compared with that of healthy controls.

The present finding of increasing serum IL-6 levels in newly diagnosed AS patients suggests the presence of an inflammatory response by the immune system against AS. These results were agrees with that obtained by Tarner *et al.* (2009) and Przepiera-Bwdzak *et al.* (2015) who found that higher IL-6 levels in serum samples of AS patients compared with healthy controls. According to these results, the level of IL-6 in blood samples was regarded as one of the important biomarkers for detecting AS.

Interleukin-6 was observed to be involved in diverse biological processes, such as the activation of T cells, induction of the acute-phase response, stimulation of the growth and differentiation of hematopoietic precursor cells, and proliferation of synovial fibroblasts results in synovial inflammation and ultimately joints damage (Choy *et al.,* 2001).

It was concluded that elevated serum IL-6 level may be a useful biomarker to detect active AS and disease progression in patients with AS (Figure 3-1).
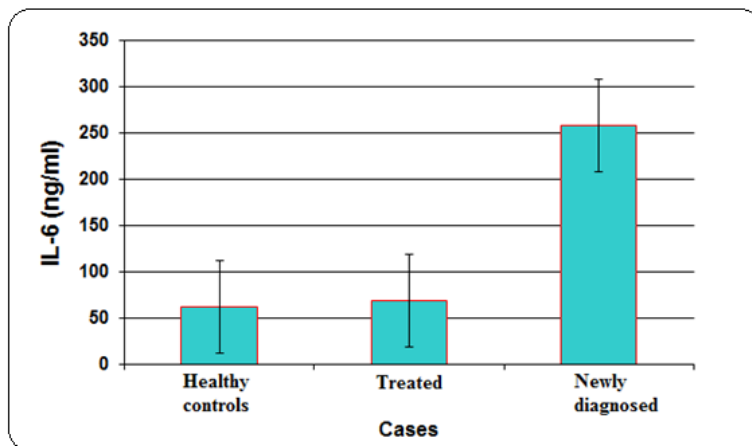
Figure (3-1): Levels of Interleukin-6 in Iraqi patients with Ankylosing spondylitis and healthy controls. The values are means of two biological replicates, and the error bars indicate standard deviation

3.2.4. Interleukin-23

IL-23 is a heterodimeric proinflammatory cytokine belonging to the IL-6/IL-12 cytokine family and it is produced by dendritic cells, macrophages, keratinocytes, and other antigen-presenting cells. IL-23 plays an important role in the pathogenesis of spondyloarthritis (Singh *et al.,* 2011).

To determine the role of IL-23 in susceptibility to AS, the levels of IL-23 in serum samples of AS patients and healthy controls was determined. Results indicated in figure (3-2) showed that the mean level of IL-23 in newly diagnosed and medically treated patients with AS was 439.25 ng/ml and 134.44 ng/ml respectively in comparison with 134.2 ng/ml in healthy controls. On the other hand, results showed that serum levels of IL-23 were significantly increased in newly diagnosed Ankylosing spondylitis patients compared with healthy controls (P<0.01) as shown in figure (3-2). Furthermore, medical treatment of those patients does not cause any significant change in IL-23 levels at a value compared with its level in healthy controls.

The significant increase in serum levels of IL-23 in newly diagnosed AS patients suggests the presence of an inflammatory response by the immune system against AS. These results were agreed with those described in many studies described by Tarner *et al* (2009), Chen *et al* (2012) and Przepiera-Bwdzak *et al* (2015) who found that higher levels of IL-23 in serum samples of AS patients compared with healthy controls.

According to these results, the level of IL-23 in blood samples was regarded as one of the important biomarkers for detecting AS, and it may be a useful biomarker to detect active AS and disease progression.
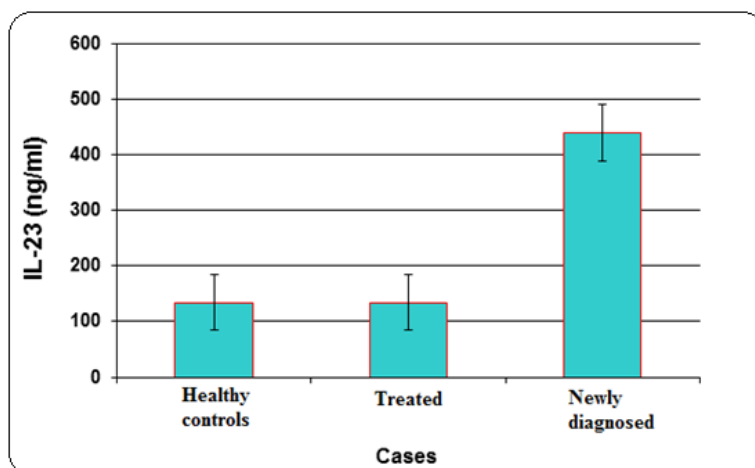

Figure (3-2): Levels of Interleukin-23 in Iraqi patients with Ankylosing spondylitis and healthy controls. The values are means of two biological replicates, and the error bars indicate standard deviation

3.3. OXIDATIVE STRESS PARAMETERS

Oxidative stress phenomenon can be found as the pathophysiological basis of chronic inflammatory joint diseases like rheumatoid arthritis and Ankylosing spondylitis, conditioning its evolving status (Kalpakcioglu and Senel, 2008). In this study, association of two oxidative stress parameters with the infection of AS were examined.

3.3.1. Serum levels of Glutathione

Glutathione (GSH) is an important non-enzymatic antioxidant factor preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella *et al.*, 2003).

Results indicated in figure (3-3) showed that the mean level of GSH in newly diagnosed and medically treated patients with AS was 1.38 ng/ml and 7.10 ng/ml respectively in comparison with 6.74 ng/ml in healthy controls.

In this study, serum levels of GSH were significantly decreased in newly diagnosed Ankylosing spondylitis patients compared with healthy controls (P<0.01) as shown in figure (3-3). Furthermore, medical treatment of those patients revealed no any significant change in GSH levels compared with that of healthy controls. The decrease in serum levels of GSH in newly diagnosed AS patients suggests the presence of an inflammatory response by the immune system against AS. These results agrees with those mentioned by Feijóo *et al* (2010) who found that lower GSH levels in serum samples of AS patients compared to healthy controls.

According to these results, the level of GSH in blood samples was regarded as one of the important biomarkers for detecting AS.

Intracellular glutathione acts as an antioxidant in several ways. It can reduce peroxides to form H_2O or scavenge directly ROS (Cuzzocrea *et al.*, 2004). It can also act as a chelator of Cu^{2+} and reduces its activity to generate ROS (Halliwell and Gutteridge, 2003). It could be concluded that decreased serum GSH level may be a useful marker to detect active AS and disease progression in patients with AS.
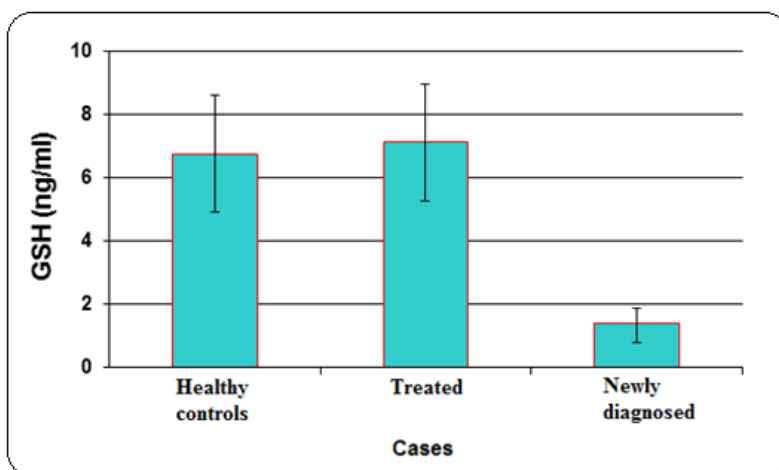

Figure (3-3): Levels of Glutathione in Iraqi patients with Ankylosing spondylitis and healthy controls. The values are means of two biological replicates, and the error bars indicate standard deviation

3.3.2. Serum levels of Malondialdehyde

Malondialdehyde (MDA) was regarded as a biomarker of lipid peroxidation and accumulation of reactive oxygen species (ROS) in Ankylosing spondylitis patients and control subjects. Reactive oxygen species play an important role in the pathogenesis of inflammatory joint diseases like rheumatoid arthritis and Ankylosing spondylitis (Ozgocmen *et al.*, 2001).

In this study, the role of MDA in susceptibility to AS was examined. Results indicated in figure (3-4) showed that the mean level of MDA in newly diagnosed and medically treated patients with AS was 1.39 nmol/L and 0.289 nmol/L respectively in comparison with 0.266 nmol/L in healthy controls. On the other hand, serum levels of MDA were significantly increased in newly diagnosed Ankylosing

spondylitis patients compared with healthy controls (P<0.01) as shown in figure (3-4). Furthermore, medical treatment of those patients revealed no any significant change in MDA levels at a value compared with healthy controls. The increase of serum MDA levels in newly diagnosed AS patients was due to the inflammatory response by the immune system against AS. These results agrees with those mentioned by Feijóo *et al* (2010) who referred that higher MDA levels in serum of AS patients was recorded compared to the healthy controls.

According to these results, the level of MDA in blood samples was regarded as one of the important biomarkers for detecting AS, and it could be concluded that the levels of MDA in serum samples is a useful biomarker to detect active AS and disease progression.
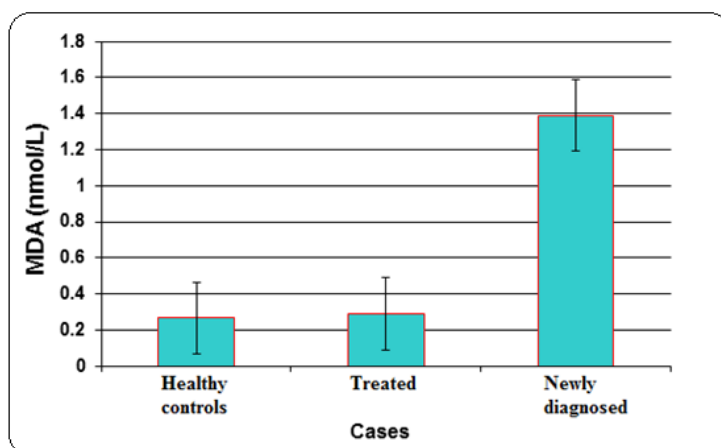

Figure (3-4): Levels of Malondialdehyde in Iraqi patients with Ankylosing spondylitis and healthy controls. The values are means of two biological replicates, and the error bars indicate standard deviation

3.4. GENOMIC DNA EXTRACTION

In order to study the genetic polymorphism in Iraqi patients with Ankylosing spondylitis, genomic DNA was extracted from blood samples of pateints and healthy controls by using ReliaPrep™ Blood gDNA Miniprep System (Promega / USA).

Results indicated in figure (3-5) showed a sharp DNA bands obtained after exraction and electrophoresis on agarose gel (0.7%). The concentration of DNA solution extracted from all samples was ranged between 200-400 μg/ml, 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 (Boesenberg-Smith *et al.,* 2012).
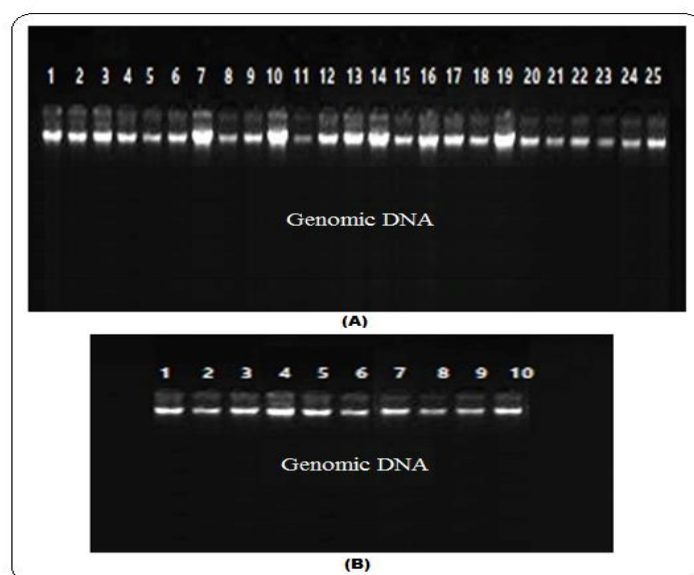

Figure (3-5): Gel electrophoresis of genomic DNA on agarose gel (0.7%) for 1 hour at 5 v/cm. (A): DNA extracted from blood sample of patients with Ankylosing spondylitis. (B): DNA extracted from blood sample of healthy controls

3.5. GENETIC POLYMORPHISM IN *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 (Zambrano-Zaragoza *et al.,* 2013). 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 (Wang *et al.,* 2012).

In this study, exon 15 of *ERAP1* was amplified in DNA solution extracted from each blood samples of patients with Ankylosing spondylitis and healthy controls. Polymerase chain reaction was achieved under optimum amplification conditions by using specific primers indicated in (2.2.4.). Results illustrated in figure (3-6) showed that the amplified products were appeared as clear bands after electrophoresis on agarose gel (2%) with 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 reagion of exon 15 for *ERAP1* gene located on short arm of chromosome 5 (Alvarez-Navarro and López de Castro, 2014). The real molecular size of this exon was 184 bp in length as mentioned in (NCBI: http://www.ncbi.nlm.nih.gov), while the molecular size of the amplified fragment was 298 bp and that's due to the nucleotide sequences of primers designed in this study which was centered on the aplification of the completed sequence of exon 15 in addition to the two border sequences located on either sides of the exon.
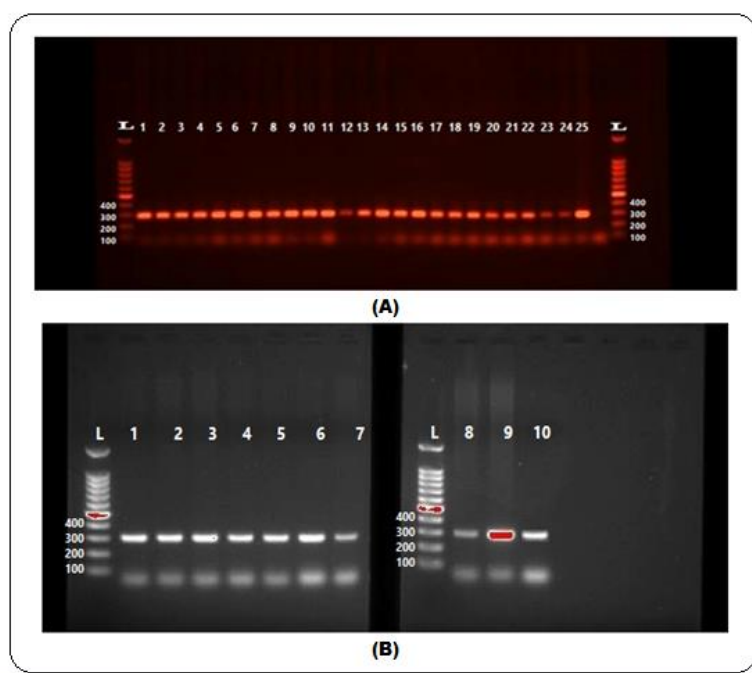
Figure (3-6): Gel electrophoresis for PCR products of *ERAP1* exon 15 on agarose gel (2%) after electrophoresis for 1 hour at 5v/cm volt in the presence of 1 kb DNA Ladder marker. (A): PCR products for amplified DNA extracted from blood samples of patients with Ankylosing spondylitis. (B): PCR products for amplified DNA extracted form blood samples of healthy controls

In this study, twenty five Iraqi patients with Ankylosing spondylitis were recruited to investigate the relationship between rs27044 SNP in exon 15 of *ERAP1* gene and the severity of the disease in compared with ten healthy controls.

3.5. Sequencing of Amplified exon 15 of *ERAP1* gene

To examine the role of the *ERAP1* SNP (rs27044) polymorphism in disease susceptibility and severity in Iraqi population with Ankylosing spondylitis,

because this (rs27044) SNP is the most important risk factor for susceptibility to Ankylosing spondylitis (Szczypiorska *et al.,* 2011). Exon 15 who was 184 base pair in length was selected to study the genetic polymorphism in this gene.

The complete nucleotide sequence of *ERAP1*, exon 15 was determined for genotyping the genetic polymorphism in this exon. Results of sequencing illustrated in figure (3-7) 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. Furthermore, the complete nucleotide sequence of this exon and the position of the expected SNP in reference sequence of *ERAP1* recorded in (NCBI Reference Sequence: *ERAP1* ID: 51752) was illustrated in figure (3-8).
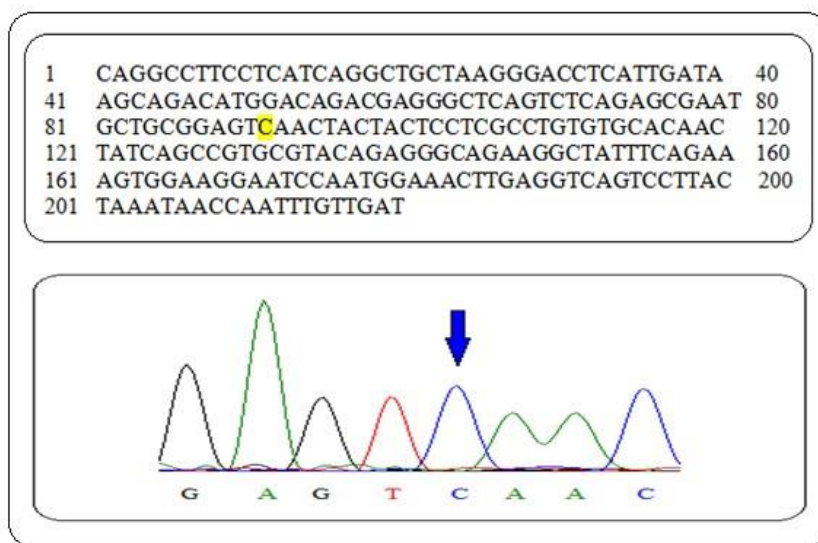
Figure (3-7): Nucleotide sequence of exon 15 of *ERAP1* in healthy controls group. Yellow letter indicates the position (96144608) of expected SNP in a sample of Iraqi population
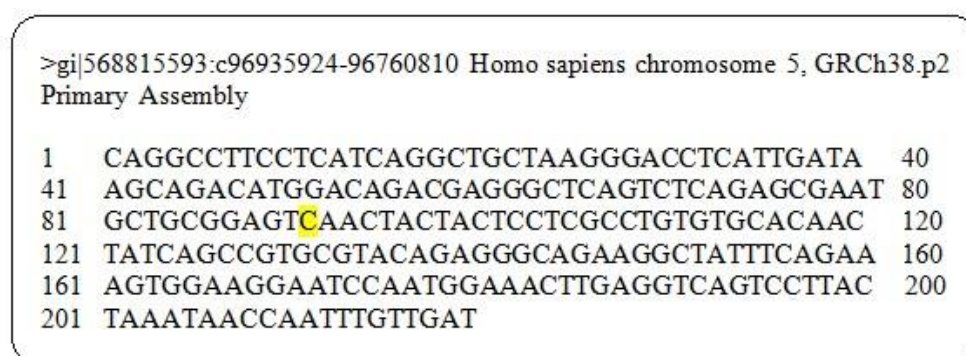
Figure (3-8): NCBI Reference sequence of exon 15 of *ERAP1* gene. Yellow letter indicated the position (96144608) of the expected rs27044 SNP

3.6. NUCLEOTIDE SEQUENCE ALIGNMENT OF THE AMPLIFIED EXON 15 OF *ERAP1* GENE

The results of the sequence analysis was analyzed by blastn 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 (3-9) and table (3-5) showed the nucleotide alignment and the position of the cytosine (C) nucleotide of healthy control group was 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
```

Figure (3-9): 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 illustrated in figure (3-10) and table (3-4) 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 (figure (3-8)). 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 (Wang *et al.,* 2012).


```
1    CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATA      40
41   AGCAGACATGGACAGACGAGGGCTCAGTCTCAGAGCGAAT      80
81   GCTGCGGAGTCAACTACTACTCCTCGCCTGTGTGCACAAC      120
121  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAA     160
161  AGTGGAAGGAATCCAATGGAAACTTGAGGTCAGTCCTTAC     200
201  TAAATAACCAATTTGTTGAT
```

Figure (3-10): Nucleotide sequence of exon 15 of *ERAP1* in non-polymorphic Ankylosing spondylitis patients group. Yellow letter indicates the position (96144608) of SNP in Iraqi population
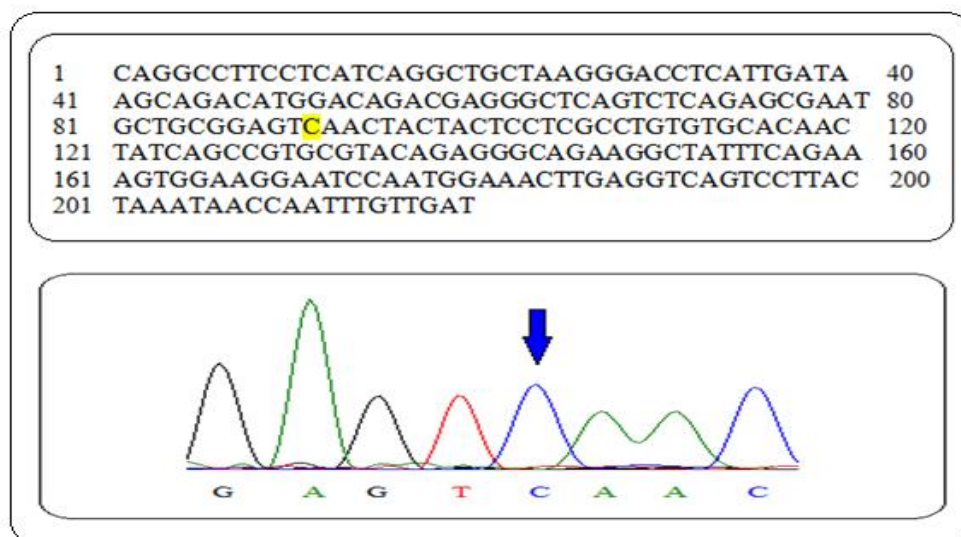
Table (3-4): Genotyping and alleles frequency in Ankylosing spondylitis patients and healthy controls

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 illustrated in figure (3-11) and table (3-5) showed the nucleotide alignment and the position of the C nucleotide (cSNP) of non-polymorphic Ankylosing spondylitis patients matched with C nucleotide in reference sequence mentioned in NCBI, showed that 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
```

Figure (3-11): Alignment between the nucleotide sequence non-polymorphic Ankylosing spondylitis patients and reference sequence. Query represents non-polymorphic patient samples; Sbject represent of database of National Center for Biotechnology Information (NCBI). Yellow letters indicates the position of matched SNP

These results were also observed in figure (3-12) that show the nucleotide sequence alignment between *ERAP1* gene, exon 15 in those patients and Iraqi healthy controls with 100% identity, from 1-220 number of nucleotide from *Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1).* This result was agreed with (Szczypiorska *et al.,* 2011) who mentioned that a nucleotide sequence of *ERAP1,* exon 15 in a sample of Ankylosing spondylitis patients was identical to healthy controls and reference sequence without occurring any genetic variation or single nucleotide polymorphism in their sequences.

Score	Expect	Identities	Gaps	Strand
407 bits(220)	1e-118	220/220(100%)	0/220(0%)	Plus/Plus

```
Query  1    CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATAAGCAGACATGGACAGACGAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATAAGCAGACATGGACAGACGAG  60

Query  61   GGCTCAGTCTCAGAGCGAATGCTGCGGAGTCAACTACTACTCCTCGCCTGTGTGCACAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGCTCAGTCTCAGAGCGAATGCTGCGGAGTCAACTACTACTCCTCGCCTGTGTGCACAAC  120

Query  121  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAAAGTGGAAGGAATCCAATGGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAAAGTGGAAGGAATCCAATGGA  180

Query  181  AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  220
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  220
```

Figure (3-12): Alignment between the nucleotide sequence for *ERAP1* (Exon 15) in Iraqi healthy controls and non-polymorphic Ankylosing spondylitis patients. Query represents healthy control samples; Sbject represent non-polymorphic patients. Yellow letters indicates the position of matched SNP

On the other hand, results illustrated in figure (3-13) and table (3-4) 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.
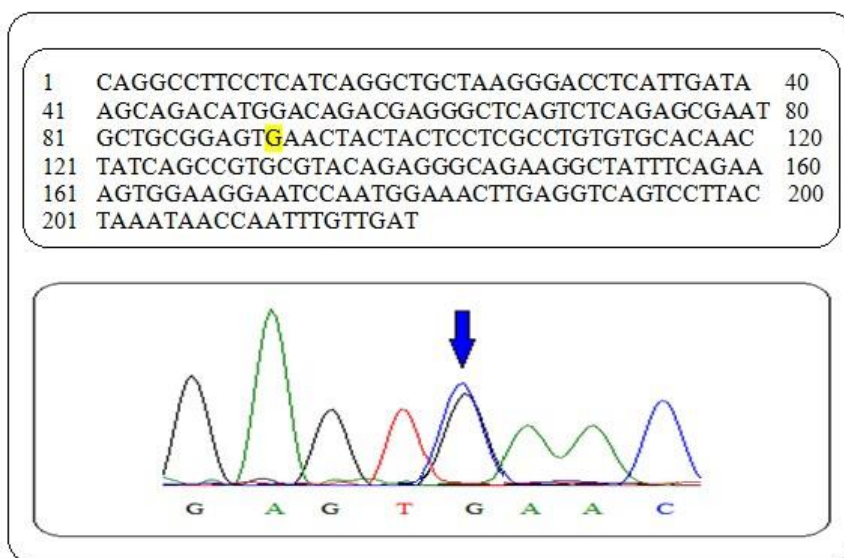


```
1    CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATA     40
41   AGCAGACATGGACAGACGAGGGCTCAGTCTCAGAGCGAAT     80
81   GCTGCGGAGTGAACTACTACTCCTCGCCTGTGTGCACAAC     120
121  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAA     160
161  AGTGGAAGGAATCCAATGGAAACTTGAGGTCAGTCCTTAC     200
201  TAAATAACCAATTTGTTGAT
```

Figure (3-13): Nucleotide sequence of exon 15 of *ERAP1* in Ankylosing spondylitis patients group. Yellow letter indicates the position of SNP in Iraqi population

Furthermore, results illustrated in figure (3-14) and table (3-5) showed the nucleotide sequence alignment and the position of the C nucleotide (cSNP) 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).*

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

Score	Expect	Identities	Gaps	Strand
401 bits(217)	2e-108	219/220(99%)	0/220(0%)	Plus/Plus

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

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

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

Query  181    AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  220
              ||||||||||||||||||||||||||||||||||||||||
Sbjct  36087  AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  36126
```

Figure (3-14): Alignment between the nucleotide sequence of polymorphic Ankylosing spondylitis patients and reference sequence. Query represents polymorphic patient samples; Sbject represent of database of National Center for Biotechnology Information (NCBI). Yellow letters indicates the position of matched SNP

These results also shown in figure (3-15), with 99% identity, from 1-220 number of nucleotide from *Homo sapiens endoplasmic reticulum aminopeptidase 1 (ERAP1)*, the cSNP allele carriers was substituted by gSNP in those 16 patients who was significantly different (P<0.01) than other nine patients who were the gSNP was not occur in the sequence of the gene exon 15 as indicated in figure (3-10).

Table (3-5): Sequencing ID in gene bank, identity, expect, score and range of DNA sequence obtained from National Center Biotechnology Information (NCBI)

No.	Accession number	Identity	Expect	Score	Range	No of samples
1	: ref|NG_027839.1|	100 %	5e-110	407	35907 to 36126	10 controls
2	: ref|NG_027839.1|	100 %	5e-110	407	35907 to 36126	9 patients
3	: ref|NG_027839.1|	99%	2e-108	401	35907 to 36126	16patients

Score	Expect	Identities	Gaps	Strand
401 bits(217)	5e-117	219/220(99%)	0/220(0%)	Plus/Plus

```
Query  1    CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATAAGCAGACATGGACAGACGAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CAGGCCTTCCTCATCAGGCTGCTAAGGGACCTCATTGATAAGCAGACATGGACAGACGAG  60

Query  61   GGCTCAGTCTCAGAGCGAATGCTGCGGAGTCAACTACTACTCCTCGCCTGTGTGCACAAC  120
            |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  61   GGCTCAGTCTCAGAGCGAATGCTGCGGAGTGAACTACTACTCCTCGCCTGTGTGCACAAC  120

Query  121  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAAAGTGGAAGGAATCCAATGGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TATCAGCCGTGCGTACAGAGGGCAGAAGGCTATTTCAGAAAGTGGAAGGAATCCAATGGA  180

Query  181  AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  220
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AACTTGAGGTCAGTCCTTACTAAATAACCAATTTGTTGAT  220
```

Figure (3-15): Alignment between the nucleotide sequence for *ERAP1* (Exon 15) in Iraqi healthy controls and polymorphic Ankylosing spondylitis patients. Query represents healthy control samples; Sbject represent polymorphic patients. Yellow letters indicates the position of matched SNP

3.7. ALIGNMENT OF EXON 15 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 (3-16a) and table (3-6) 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 (3-16b) and table (3-6) also 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 as showed in figure (3-11).

Amino acid alignment between Iraqi patients with Ankylosing spondylitis (gSNP) and reference *ERAP1*, under sequence ID: pdb|3RJO|A, was shown in figure (3-16c) and table (3-6). 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 (3-7) 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 (Zambrano-Zaragoza *et al.,* 2013). This alteration in ERAP1 structure may cause the evaluation of cytokines levels (Interlukin-6 and Interlukin-23) (Cui *et al.,* 2003) 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 as it was mentioned by (Harvey *et al.,* 2009).
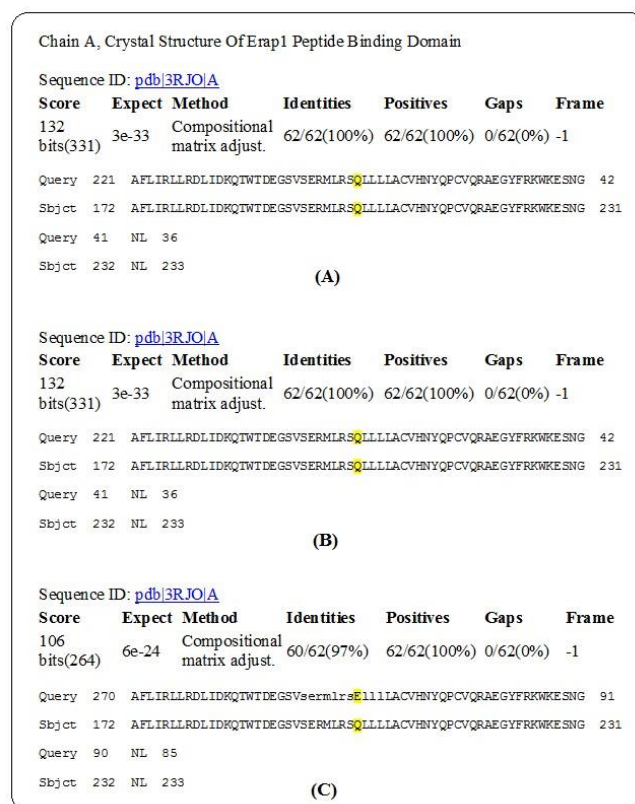

Figure (3-16): Alignment of amino acid encoded by exon 15 of *ERAP1* gene in Ankylosing spondylitis Iraqi patients (Query) and with the reference sequence (Sbject) scored by National Center Biotechnology Information (NCBI). Yellow letters indicates the position of the expected polymorphic amino acid. (A): Healthy controls. (B): Non-polymorphic patients. (C): Polymorphic patients

These results agree with those obtained by (Szczypiorska *et al.,* 2011) who mentioned that cSNP substituted with gSNP in a sample of Spanish population.These results also agree with those obtained by (Wang *et al.,* 2012) who mentioned that cSNP substituted with gSNP in a sample of Taiwanese population.

Table (3-6): Sequencing ID in gene bank, identity, range and expect of amino acid sequence obtained from National Center Biotechnology Information (NCBI)

No.	Accession number	Identity	Range	Expect
1	: pdb|3RJO|A	100 %	172-232	3e-33
2	: pdb|3RJO|A	100 %	172-232	3e-33
3	: pdb|3RJO|A|	97%	172-232	6e-24

Table (3-7): 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

References:

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