GENETIC POLYMORPHISMS OF *HLA-G* GENE IN RHEUMATOID ARTHRITIS

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ABSTRACT

This study was aimed to evaluate the influence of Human Leukocyte Antigens- G (*HLA-G*) gene polymorphisms on the risk of rheumatoid arthritis (RA). In the present study, blood samples were collected from 100 rheumatoid arthritis patients and 100 healthy controls. Serum levels of HLA-G in both patients and controls were measured using ELISA kit which was shown a higher significant differences ($p< 0.05$) in comparison between them (539.3 ng/ml and 139.3ng/ml) respectively. The frequency of the allele and genotypes in the patient groups and control groups were determined using Polymerase Chain Reaction (PCR) for 14 bp polymorhisms in exon 8 (rs66554220) while direct sequencing analysis was done to determine exon 2 (rs1130355) (G 1850 A) SNPs of human leucocyte antigens- G (*HLA- G*). There was statistically significant difference in all genotypes of *HLA-G*14-bp of exon 8 in RA patients in comparison with controls. The probability of homozygote genotype of insertion was (0.006), homozygote genotype of deletion was (0.003) and for heterozygote of insertion was (0.000) in comparison between RA patients and controls. From the results of odd ratio (OR) found that existence of homozygotes of insertion give protection effect from disease O.R= 0.44 while heterozygotes of deletion act as risk factor for disease O.R= 3.31. Homozygotes of deletion had no effect because was not found in patients groups. From the sequencing results of exon 2 of *HLA-G* gene found the mutation occur in position 1850 G<A which give three genotypes (GG, AG and AA). Calculations of genotypes frequency for exon 2 showed that GG and AA genotypes was associated with the disease with *p*- value (0.000) for both genotypes. While AG genotype showed no association with disease *p* value (0.449).From the results of odd ratio found that GG and AA of exon 2 gave protection effect from disease O.R(0.29 and 0.76) respectively, while AG genotype acted as risk factor for disease O.R= 4.0.

Keywords: autoimmune disease, 14bp insertion / deletion, exon 2, exon 8

تعدد الاشكال الوراثية لجين *HLA-G* في التهاب المفاصل الرثوي

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المستخلص

تهدف الدراسة لتقييم تأثير الأشكال الوراثية لجين *HLA-G* على خطر الإصابة بالتهاب المفاصل الرثوي (RA). في هذه الدراسة تم جمع عينات الدم من 100 مريض مصاب بالتهاب المفاصل الرثوي و 100 من الاصحاء. تم قياس مستويات مصل HLA-G في كل من المرضى والسيطره باستخدام طريقة ال ELISA التي أظهرت فروق ذات دلالة إحصائية أعلى ($p <0.05$) عند المقارنة بينهما (539.3 نانوغرام / مل و 139.3 نانوغرام / مل) على التوالي. تم تحديد تردد الأليل والأنماط الجينية في مجموعات المرضى ومجموعات السيطرة باستخدام تفاعل البوليميريز المتسلسل لتعدد الأشكال 14 زوج / قاعدة في إكسون 8 (rs66554220) بينما تم إجراء تحليل التسلسل المباشر لتحديد SNPs (G 1850 A) (rs1130355) exon 2 لـ مستضد كريات الدم البيضاء البشرية HLA – G. كان هناك فروق إحصائيًه في جميع الأنماط الجينية لـ HLA-G14-bp من exon 8 في مرضى RA مقارنةً بمجموعة السيطرة. كان احتمال النمط الجيني متماثل الزيجات للاضافة =(0.006) ، وكان النمط الجيني متماثل الزيجات للحذف =(0.003) و متغاير الزيجات اضافة كان (0.000) مقارنة بين مرضى التهاب المفاصل الرثوي والسيطرة. من نتائج نسبة الاحتمالية (OR) وجدت أن وجود متماثلة الزيجات للاضافة يعطي تأثير الحماية من المرض OR = 0.44 بينما تعمل الزيجات المتغايرة كعامل خطر للإصابة بالمرض OR = 3.31. من نتائج تسلسل exon 2 لجين *HLA-G* وجد أن الطفرة تحدث في الموضع 1850 G> A الذي يعطي ثلاثة طرز وراثية (GG و AG و AA). أظهرت نتائج تردد الطرز الوراثية لـ exon 2 أن الطرز الوراثية GG و AA ارتبطت بالمرض بقيمة p (0.000) لكلا الطرازين. بينما لم يظهر التركيب الوراثي AG أي ارتباط مع قيمة p المرض (0.449). من نتائج نسبة الاحتمالية وجدت أن GG و AA من exon 2 أعطت تأثيرًا وقائيًا من المرض OR (0.29 و 0.76) على التوالي ، في حين أن التركيب الوراثي AG كان بمثابة عامل خطر للمرض اذ كانت نسبة الاحتمالية (OR) = 4.0.

الكلمات المفتاحية: امراض المناعة الذاتية , 14 زوج قاعدة اضافة/حذف ,اكسون 2 , اكسون 8

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INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory chronic autoimmune disease affecting of the connective tissues characterized by inflammation and degradation of the joints. It affects 0.5–1.0 percent of world's population , mostly between the ages of thirty–fifty years, it is two to four times more prevalent in females than in males. Patients with RA characterized typically present with pain and stiffness in multiple joints, and swelling caused by synovial inflammation (5). Fever, tiredness, nodules, interstitial lung involvement, vasculitis, anemia, and osteoporosis are possible additional symptoms. A rise in acute-phase reactants such C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) is also possible (31). The illness may worsen, causing joint injury, distortion, incapacity, and even death. Rheumatoid arthritis is caused by an unknown etiology , however it is thought to be caused by reaction between genetic environmental factors and infectious, with a triggering event., Inflammation in the joints, possibly due to an autoimmune or infectious response, causes a complicated immunological response that eventually leads to rheumatoid arthritis complications. Rheumatoid arthritis patients' bone marrow mononuclear cells have in the immune response, abnormal regulatory networks (21). *1910 genes were found to be downregulated and 764 genes to be increased in bone marrow-derived rheumatoid arthritis mononuclear cells, including the HLA-G gene. Using genome-wide association studies (GWAS), it was confirmed that the HLA-G gene, which is found on the short arm of chromosome sex within the HLA region, is linked to rheumatoid arthritis. It has seven introns and eight exons that code for the heavy chain of the HLA-G protein*. Because exon sex contains a stop codon, exons seven and eight are always absent in mature mRNA (11). Seven expressed isoforms was identified, four of which are membranous (HLA-G1-G4) and three of which are soluble molecules (HLA-G5-G7) (27). The soluble HLA-G1 form is generated by proteolytic cleavage of the HLA-G1 isoform (14). Compared to the conventional HLA molecules, the HLA-G gene has just forty-sex segregating variants that can be mapped to either the coding or non-coding regions. Only fifteen variations were observed at the protein expression level (16). Polymorphisms in non-coding regions, specifically these in the 3' untranslated regions and (3' UTR) 5' upstream regulator region (5' UTR) , are thought to affect the function of human leukocyte antigen-G molecules and was linked to a variety of diseases, including rheumatoid arthritis infertility preeclampsia and in vitro fertilization failure (19). The importance of the 3' UTR in the expression profile of human leukocyte antigen-G has been proven by many research (13, 22). Numerous regulatory elements, including as AU-rich motifs and poly-A signals, are present in this area and play a role in mRNA stability, isoform alternative splicing patterns, and maybe HLA-G function. Attention has been drawn to the 14 base pair (bp) insertion/deletion (indel) polymorphism that maps to location 3741 in the 3' UTR of exon eight (1). Numerous studies have revealed a link between the indel polymorphism and HLA-G mRNA stability and splicing patterns that result in the development of HLA-G isoforms (9, 30). In addition, the 14 bp insertion allele has been associated with low levels of HLA-G mRNA as well as circulating soluble HLA-G (sHLA-G) isoforms (4,9). Plasma concentrations of sHLA-G with the genotype are also lower +14bp / +14bp than with +14bp / -14bp and -14bp/-14bp genotypes (9,22). As a result, the HLA-G molecule is thought to be a significant role in early and late rheumatoid arthritis, To understand the importance of HLA-G in rheumatoid arthritis, There have been various research on gene polymorphisms and protein expression. The genetic analysis of the human leukocyte antgen-G 14 bp insertion/deletion polymorphism and two promoter SNPs (rs2735022, -586C/T and rs1736936, -1202T/*C*) in *HLA-G* gene found there no differences were allelic or genotypic in rheumatoid arthritis patients classified by disease development and characteristics (20). contrariwise , study of patients with juvenile idiopathic arthritis (JIA), When compared to controls of the same gender, In females, there was a significant association between the 14 bp DEL allele and susceptibility to juvenile

idiopathic arthritis (16). These information eliminate any insinuation of *HLA-G* genetic background in rheumatoid arthritis, but they do support a possible role in juvenile idiopathic arthritis. These explanations support the presence of different physio pathogenic pathways between rheumatoid arthritis and juvenile idiopathic arthritis. Furthermore, rheumatoid arthritis and juvenile idiopathic arthritis present different HLA associations, this indicates that the immune influences involved in susceptibility to these two illnesses are different . The serum concentration of HLA-G (sHLA-G) is significantly decreased both in rheumatoid arthritis and in juvenile protein Idiopathic Arthritis (28,23). patients as compared to controls A comparable reduction in serum HLA-G concentrations may result in persistent chronic activation inflammatory cells, which may contribute to the development of those two illnesses . Low sHLA-G levels are unable to sustain an an inflammatory and immune regulated systemic environment thereby worsening disease progression. Despite the fact that female susceptibility to juvenile idiopathic arthritis corresponds with the high producer 14 bp DEL allele, HLA-G secretion in the serum is limited . Compared with the genetic background, these data suggest a stronger effect of protein transduction mechanisms . Higher levels of sHLA-G molecules were found in both rheumatoid arthritis and juvenile idiopathic arthritis patients when sHLA-G molecules were measured at the synovial inflammatory site (15,24). The recruitment and local synthesis by active synovial fibroblast of activated HLA-G positive immune cells that can interact in the form of immune inhibitor receptors and promote the persisting inflammatory response, could be related to HLA-G release into inflamed synovium . The levels of sHLA-G are greater and show a positive link with disease activity indices including C-reactive protein (CRP) and the number of swollen joints in rheumatoid arthritis patients with HLA specificities associated with the illness (HLA - DRB1 *01, HLA - DRB1 *04, and HLA - DRB1 *10). (28). Incapacity to control inflammation can be shown in the higher serum HLA-G level and upregulation detected with profile analysis of the *HLA-G* gene transcription (21). These date propose that HLA-G molecules are produced differently depending on the native and systemic environments which are characterise by various chemical factors and cell types. Importantly more study is needed to determine the important of HLA-G molecules in development of rheumatoid arthritis. In this study we determined serum level of HLA-G in patients and controls and examined the association between 14 bp ins/del polymorphism of exon 8 and the polymorphism in exon 2 of *HLA-G* gene with RA patients in contrast with controls in Iraqi population.

MATERIALS AND METHODS

Patients and controls: Peripheral blood samples (10 mL) were collected into two tubes after receiving the written informed consent of all study participants and approval from the local ethics commission. one with EDTA for genetic analysis and other without EDTA for getting serum to measure HLA-G concentration from 100 patients with RA and 100 controls , stored at −20 °C until used.

Measurement of HLA-G Concentrations: The obtained serum that stored at –20°C was used to measure the HLA-G concentration in serum samples using ELISA sandwich kits (Shanghai biological, China) following the manufacturer's instructions.

DNA extraction: Total genomic DNA from both groups organizations had been collected in tubes with EDTA was extracted by ReliaPrep™ Blood gDNA Miniprep system kit (promega, USA) and DNA was resuspended in RNase/DNase -free distilled water after following the manufacturer's instructions. The purity and concentration of genomic DNA were determined using Nanodrop UV/VIS (2). The DNA samples were stored at −20 °C until used.

Determination of 14-bp of Exon 8 in *HLA-G* gene: Exon 8 of the *HLA-G* gene was amplified by polymerase chain reaction (PCR) using the primers GE14HLAG (5'-GTGATGGGCTGTTTAAAGTGTCACC-3') and RHG4 (5'-GGAAGGAATGCAGTTCAGCATGA-3')(13). The PCR procedure included an initial denaturation phase at 94° C for 5 minutes and a final expansion of 35 cycles for 20 s at 94°C, 30 s at 64°C and 60 s at 72°C for 10 minutes at 72°C. A 3 percent agarose gel, stained with ethidium bromide, was analysed (210 / 224 bp),

using the gel documentation system, in fragment sizes of PCR products depending on presence or absence of a specified band.

Determination of Exon 2 polymorphism in *HLA –G* gene: The DNA samples were amplified for exon 2 by using a pair of specific primers Exon 2: Forward: 5`-TCCATGAGGTATTTCAGCGC-3`, Reverse: 5`- CTGGGCCGGAGTTACTCACT-3` as stated by (26). Using the polymerase chain reaction (PCR), the following PCR cycle conditions were used in a total volume of (25 µl): Initial denaturation temperature was 95C for 5 minutes, followed by 35 cycles of denaturation for 30 seconds, annealing for 30 seconds, extension for 30 seconds, and final extension for 5 minutes at 72C. Electrophoresis on a 1.5 percent agarose gel resolved the PCR products and the ladder marker. The gel was viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain. After 2 hours of electrophoresis at 70 Volt, the molecular size of the bands was estimated using a DNA ladder (a 100bp DNA ladder). Statistical analysis: The number of genotype and allele frequencies were investigated using the Hardy-Weinberg equilibrium (HWE) (18), The Chi-square test was used to compare genotypes in each sample. The risk or benefit effect of the researched factor between groups was assessed using the odds ratio and confidence intervals. The direct counting approach was used to calculate frequencies of the HLA-G 14 bp (indel) polymorphism. With the SAS software version 9.1 ed SAS Inst. INC. Cary. N.C. USA) , differences between populations were examined using T-test.

RESULT AND DISSCUSSION

In this case-control study, we looked at the serum level, allele distribution, and genotypes of *HLA-G* 14 bp indel (rs66554220) polymorphism and exon 2 (rs1130355) (G 1850 A)SNPs among RA and healthy controls. 100 rheumatoid arthritis and 100 healthy subjects were recruited to this study.

Serum levels of HLA-G in patients with Rheumatoid arthritis: Serum levels of HLA-G was showed a higher significant differences (p<0.05) in patients compared with healthy control (539.3 ng/ml and 139.3ng/ml) respectively. The first significant conclusion is that in RA patients circulation SHLA-G levels have been increased. This corresponds to the results from (25) It found that all early untreated rheumatoid arthritis patients had trace levels of sHLA-G in plasma comparing with minority 23 percent of healthy controls, which were raised in those patients after rheumatoid arthritis therapy. This rise could reflect an attempt of the immune system to counter balance the autoimmune process. Our results are at variance to the report on serum HLA-G in rheumatoid arthritis showing lower plasma HLA-G levels in rheumatoid arthritis patients in comparison with healthy control (15).

Determination the exon 8 in *HLA-G* gene: Electrophoresis was used to detect the 14 bp deletion polymorphism. The amplified PCR products were either of 210 or 224 bp depending on the deletion of the 14 bp from exon-8. After the gel was stained with ethidium bromide stain, the PCR product was seen and scored using a UV transilluminator. (Figure 1). By the X2 analysis differences were revealed between the two groups (Hardy –Weinberg equilibrium).

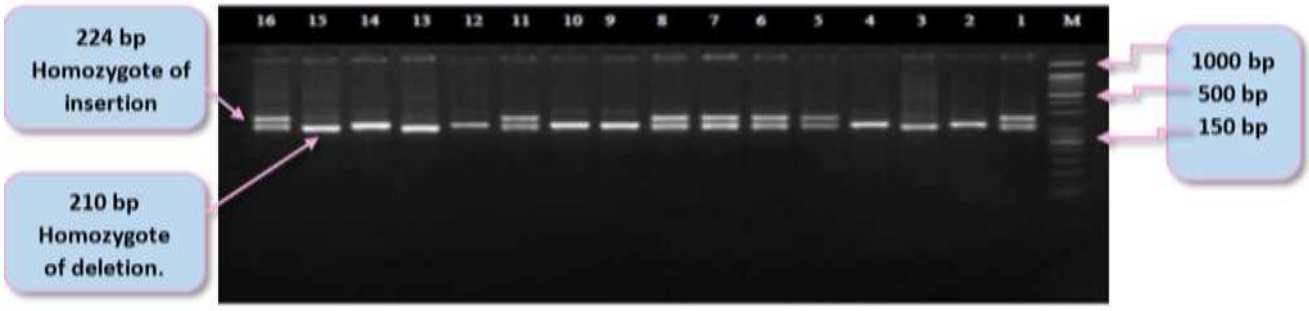

Figure 1. Detection of 14- bp) INDEL polymorphism of *HLA-G* on 3% agarose gel. Gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain. Lane M: 25/100 bp mixed DNA marker, Lane 1, 5-8, 11, 16: Heterozygote for deletion, Lane 2-4, 9, 10, 12-15: Homozygote for deletion

In this study base, we looked at the distribution of genotypes and alleles for the exon 8 of the HLA-G gene's 14 bp ins/del polymorphism among Iraqi patients with rheumatoid arthritis and healthy controls. The genotype distribution of the 14bp polymorphism in rheumatoid arthritis was not in Hardy Weinberg equilibrium ($p < 0.05$); Compared to expectations more heterozygotes have been detected. This shows that 14 bp HLA-G polymorphism may has some important for the development of RA . There are many heterozygotes of 14 bp for the (ins/del) sequence in rheumatoid arthritis compared with healthy subject. While homozygote for deletion (del/del) sequence was absent from rheumatoid arthritis patients. The distributions of allele and genotype frequencies of *HLA-G* 14bp ins/del polymorphisms are reported in table (1 and 2). In this study we found there was high significant differences between genotypes in rheumatoid arthritis patients in comparison with control groups $p< 0.05$. The frequency of 14 bp (+14bp/+14bp genotype) in rheumatoid arthritis was (33.0%), while the frequency of 14 bp (+14bp/+14bpgenotype) in control group was (53.0%), and the odd ratio was 0.44(95%CI=0.24-0.80) for both groups. The estimated frequencies of 14bp (+14bp/-14bp) genotype in rheumatoid arthritis was (67.0%), while the frequency of 14bp (+14bp/-14bp) genotype in control group was (38.0%), and the odd ratio was 3.31(95%CI=1.78-6.17). The significance analysis was higher in both 14bp (+14bp/+14bp and +14bp/-14bp) genotypes which were ($p< 0.006$ and $p< 0.000$), respectively. The significance of such association was assessed Fisher`s Exact Probability. Such assessment is more preferred, because it allows for correction of probability and it is not affected by small numbers (less than 5). It seems that the chance of prevent the disease occur is greater when the patients are homozygote of insertion (odd ratio of (+14bp/-14bp) was 0.44) rather than heterozygote (odd ratio of (+14bp/- 14bp) was (4.39). This could be reflection of the link between (+14bp) sequence and altered balance in the isoforms and isoforms of HLA-G mRNA (probably with protein). Homozygotes of insertion rather than heterozygotes genotypes may be an benefit for the species because may contribute to avoiding the rheumatoid arthritis. The genotype allocation of the 14 bp polymorphism in rheumatoid arthritis dose not in Hardy Weinberg equilibrium ($p <0.05$). Five requirements must be satisfied to establish equilibrium: The population should be large. this population should be isolated from other populations, alleles mutations not happened (DEL/INS), random marriage and no natural selection. If five requirements are not achieved, evolution takes place and the allele frequency in the population and Hardy Weinberg equilibrium is not present (12). Because the mutation occurred in allele by deletion 14 bp (heterozygote) in this study and inbreeding (increased recessive alleles) may occurred that led to deviate in Hardy - Weinberg equilibrium and the genotypes distribution were not in this equilibrium. This result was agreed with (8) that the genotype distributions of the 14 bp polymorphism in controls were not in Hardy-Weinberg equilibrium. This 14 bp polymorphism has been linked to HLA-G mRNA isoforms that lacked the first 92 base sequences in exon-8 (3' UTR) (10). The 14 bp sequence at the start of exon-8 may be responsible for the HLA-G transcript's alternative splicing. This sequence may operate as a cryptic branch point sequence for mRNA splicing throughout mRNA processing.

Table 1. Allele and genotype frequencies and Hardy-Weinberg equilibrium of 14 bp insertion deletion polymorphism of *HLA-G* gene in rheumatoid arthritis patients and controls

Groups			14-bp Insertion/Deletion Polymorphism Genotypes			HWE $p\leq$	Alleles	
			ins/ins	ins/del	del/del		del	ins
patients (No. = 100)	Observed	No.	33	67	0	0.00	67	133
		%	33%	67%	0%		33.5%	66.5%
	Expected	No.	44.22	44.65	11.22		Not estimated	
		%	44.22%	44.65%	11.22%			
Controls (No. = 100)	Observed	No.	53	38	9	0.565	56	144
		%	53%	38%	9%		28%	72%
	Expected	No.	51.84	40.32	7.84		Not estimated	
		%	51.84%	40.32%	7.84%			

HWE: Hardy-Weinberg Equilibrium

Table 2. Observed numbers and percentage frequencies of exon 8 (INS/DEL) in *HLA-G* gene in rheumatoid arthritis patients and controls

Genotypes		Patients (No.=100)		Controls (No.=100)		Odd Ratio	95% C.I.	*p*- value
		No.	%	No.	%			
Ins/ins (homozygous of insertion)		33	33%	53	53%	0.44	0.24-0.80	0.006
Ins/del (heterozygous of insertion)		67	67%	38	38%	3.31	1.78-6.17	0.000
Del/del (homozygous of deletion)		0	0%	9	9%	0.00	0.00-0.48	0.003
Total		100	100%	100	100%	-	-	-
Allele frequency	*ins*	133	66.5	144		1.30	0.83-2.03	0.279
	del	67	33.5	56		0.77	0.49-1.21	0.279

Determination the exon 2 in *HLA-G* gene

Exon 2 was amplified using the primers as shown in materials and methods. After staining the gel with ethidium bromide stain, the PCR product was imagined and scored using a Gel documentation UV trans illuminator (Figure 2)

Figure 2. Electrophoresis on a 1.5 percent agarose gel was used to separate the PCR products from the ladder marker. The gel was dyed with ethidium bromide stain before the electrophoresis, which was carried out at 70 volts for two hours, was began. The molecular size of the bands was calculated using a DNA ladder (100bp ladder).. PCR products of exon 2 of *HLA-G* gene of the molecular size 281 bp. M: Marker, lanes 1-5: control samples, lanes 6-18: patients sample

Among many polymorphisms found in *HLA-G* selected from databases single-nucleotide polymorphism A>G in Exon 2 in position 1850 was investigated for association with rheumatoid arthritis in Iraqi patients in this case-control study (100 cases and 100 control). PCR product for the specific amplified region was sequenced to determine allele frequencies

for the expected SNP, results illustrat in (Figure 4) show the nucleotide sequence of the specific region including 1850 in rheumatoid arthritis patients and healthy controls.

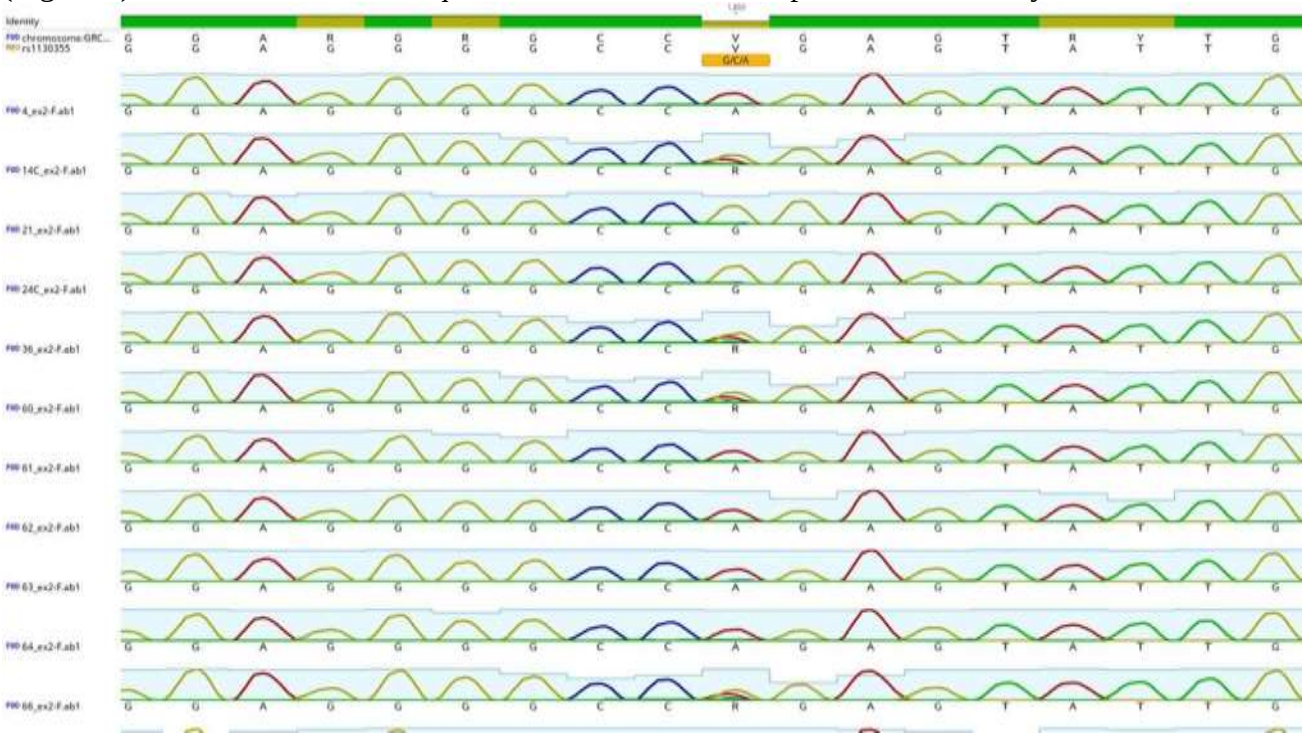

Figure 3. Nucleotide sequence of the specific region of Exon2 for rheumatoid arthritis cases, letter R represents heterozygote (AG), letter G for wild homozygote (GG) and A for mutant homozygote (AA), the results analyzed by genous software

The nucleotide sequence was analyzed by combining data from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) with our data. The SNP in *HLA-G* was genotyped in selected group of controls and rheumatoid arthritis patients. Results of alignment illustrat in (Figure 4) show the position of expected SNP (Adenine nucleotide) in position 1850 at exon2 of *HLA-G*, the nucleotide sequence was 99% similar to the reference sequence mentioned in NCBI under ID: MN795163.1 for *Homo sapiens HLA-G* gene.

Homo sapiens MHC class I antigen (HLA-G) gene, HLA-G*01:01:01:13 allele, complete cds

Sequence ID: MN795163.1 Length: 2864 Number of Matches: 1

Range 1: 286 to 506 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
405 bits(219)	7e-109	220/221(99%)	0/221(0%)	Plus/Plus

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Query  1    CCCGCTTCATCGCCATGGGCTACGTGGACGACACGCAGTTCGTGCGGTTCGACAGCGACT  60
Sbjct  286  ............................................................  345

Query  61   CGGCGTGTCCGAGGATGGAGCCGCGGGCGCCGTGGGTGGAGCAGGAGGGGCCRGAGTATT  120
Sbjct  346  ...........................................................G..  405

Query  121  GGGAAGAGGAGACACGGAACACCAAGGCCCACGCACAGACTGACAGAATGAACCTGCAGA  180
Sbjct  406  ............................................................  465

Query  181  CCCTGCGCGGCTACTACAACCAGAGCGAGGCCAGTGAGTAA  221
Sbjct  466  ........................................  506
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Figure 4. Alignment of exon2 sequence of subject that shows the presence of Guanine nucleotide, query represents healthy that aligned with the reference sequence(subject).

There are three genotypes (GG, AG, AA) and two alleles available for the SNP of the HLA-G gene (G>A; found on Chromosome 6). (G and A). The genotypes were not consistent with the Hardy-Weinberg equilibrium (HWE) in the healthy and rheumatoid arthritis groups, and significant disparities ($p > 0.05$) were indicated between the observed and expected genotype frequencies in both groups table (3).

Table 3. Exon 2 polymorphism of the *HLA-G* gene allele and genotype frequencies, and Hardy-Weinberg equilibrium, in rheumatoid arthritis patients and controls

Groups			Exon2 Polymorphism Genotypes			HWE $p \leq$	Alleles	
			GG	*AG*	*AA*		G	A
patients (No. = 100)	Observed	No.	18	29	53	0.000	65	99
		%	18%	29%	53%		32.5 %	67.5%
	Expected	No.	10.56	43.88	45.56		Not estimated	
		%	10.56%	43.88%	45.56%			
Controls (No. = 100)	Observed	No.	43	35	22	0.007	121	79
		%	43%	35%	22%		60.5 %	39.5%
	Expected	No.	36.6	47.8	15.6		Not estimated	
		%	36.6%	47.8%	15.6%			

HWE: Hardy-Weinberg Equilibrium

Inspecting *HLA-G* gene genotypes and allele frequencies in rheumatoid arthritis and control groups revealed that there were a significant variation in the homozygous of wild type and mutant type $p< 0.01$, while in heterozygous mutant type there was no significant differences $p> 0.05$, although decreased frequencies of G and A alleles were observed in patients compared to control groups (Table 4). In exon 2 Polymorphism, the odds ratio for the GG genotype was 0.29 (0.14 - 1.85) with p=0.000, the odds ratio for the AG genotype was 0.76 (0.40-1.44)with p=0.449, and the odds ratio for AA genotype was 4.0 (2.08-7.79) with p=0.000 indicating that heterozygous genotype AG and was the risk factor for disease, while homozygous genotypes AA, GG was considered protected factor from disease.

Table 4. Observed numbers and percentage frequencies of exon2 in *HLA-G* gene in rheumatoid arthritis patients and controls

Genotypes		Patients (No.=100)		Controls (No.=100)		Odd Ratio	95% C.I.	*p*- value
		No.	%	No.	%			
GG (homozygous wild type)		18	18%	43	43%	0.29	0.14-0.58	0.000
AG (heterozygous mutant type)		29	29%	35	35%	0.76	0.40-1.44	0.449
AA (homozygous mutant type)		53	53%	22	22%	4.0	2.08-7.79	0.000
Total		100	100%	100	100%	-	-	-
Allele frequency	*G*	65	32.5%	121	60.5%	0.31	0.20-0.48	0.000
	A	135	67.5%	79	39.5%	3.18	2.07-4.90	0.000

Without coming to a definitive consensus, numerous studies have looked into the significance of HLA-G polymorphisms in rheumatoid arthritis susceptibility. two SNPs (rs1736936, 1305 G/A, and rs2735022, 689 A/G) were examined. There is no proof that the HLA-G promoter contributes to the onset of rheumatoid arthritis in the Korean population (14). The findings in a Brazilian population demonstrated the importance of 3′ UTR polymorphisms in rheumatoid arthritis follow-up (7). The authors observed a substantial relationship between the −762C > T, −716T > G, −689A > G, −666G > T, −633G > A, −486A > C,and −201G > A (rs1632946; rs2249863; rs273502 2; rs35674592; rs1632944; rs1736933; and rs1233333) SNPs with disease. The 14 bp deletion allele is associated with female susceptibility to juvenile idiopathic arthritis, according to an examination of 106 patients with the disease. These numerous associations imply that rheumatoid arthritis, juvenile idiopathic arthritis, and have a number of pathogenic components in common (6). and children with juvenile idiopathic arthritis have

lower serum HLA-G concentrations than controls (24), which may be related to the inflammation's chronic nature. A marker for the treatment of rheumatoid arthritis has also been investigated using the HLA-G14bp insertion/deletion polymorphism. In brief, we suggested that HLA-G 14 bp insertion/deletion and exon 2 (rs1130355) (G 1850 A) SNPs in mutant type is a candidate gene that confers the genetic susceptibility for rheumatoid arthritis development in Iraqi population.

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