HLA-G Gene Polymorphism and Soluble HLA-G Serum Level in Rheumatoid Arthritis Patients under Treatment with TNF-γ Antagonists

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Abstract
Objective: - Aim of the study to investigate the association between HLA-G gene polymorphism and soluble HLA-G serum level with rheumatoid arthritis patients on biological drugs. Materials and Methods: - HLA-G (rs1063320) SNP was genotyped and measurement of serum level was done in 100 RA patients on biological treatment (Etanerecept (Enbrel) or Infliximab) from 4 to 6 months and in 100 healthy controls. HLA-G SNP was analyzed by SYBR Green-based quantitative real-time polymerase chain reaction (qPCR) allelic specific assay. Serum level of soluble HLA-G investigate by ELIZA technique. Results: - There was a significant association between the HL-G (rs1063320) SNP with risk to develop RA (P value<0.001). Also, soluble HLA-G serum level was lower in patients with RA than control but statistically not significant (p value 0.121), but significant relationship with DAS28 p=0.01. Conclusion: - HLA-G (rs1063320) SNP may be risk factors to develop RA. Positive association between serum level of sHLA-G and DAS28.
Keywords: - HLA-G, soluble HLA-G, Rheumatoid arthritis, biological drugs
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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that severely affects the life quality of patients. It is estimated that approximately 1% of the people around the world suffers from RA (1). The disease is 2-3 times more common in females than in males. It has been proposed that both genetic and environmental factors are involved in the expression and complications of the disease (2, 3). Genetic factors are assumed to contribute to up to 60% of the risk of developing RA (4). Biological agents block certain chemicals in the blood from activating the immune system and henceforward protect patients' joints (5).
HLA-G is a nonclassical HLA class I molecule that can bind to immune cells and inhibit their function (6, 7). The gene is found inside HLA locus on chromosome six. HLA-G products show some unusual features for which they are considered as non-classical HLA-I antigens, the restriction of their allelic polymorphism, the expression of seven isoforms characterized by four membrane-bound (G1, G2, G3, and G4) and three soluble (G5, G6, and G7) proteins and, the limitation of their tissue distribution (8).
It is involved in several immunoregulatory processes and may potentially be involved in the pathogenesis of RA. Genetic variants in coding and noncoding regions of the HLA-G may affect biological features of the molecule. Expression rate of HLA-G gene and plasma level are affected by variants in the promoter region as well as 3'UTR (9).

2. Materials and Methods

Subject

In this case-control study one hundred (100) patients with Rheumatoid Arthritis were enrolled. Their age ranged from 23 to 58 years. They were collected from Rheumatology unit in Baghdad Teaching Hospital from November 2018 to August 2019. The diagnosis of each case was done by a rheumatologist confirmed by laboratory investigations. They were classified according to the criteria of EULAR/ACR (2010). Patients received anti-TNF therapy Etanercept (Enbrel) (64 patients) or infliximab (36 patients) from 4 to 6 months. Nine patients were responder to biological drugs and (91) were nonresponders. Control group included 100 apparently healthy volunteers whose ages and sex were matched with patients group. **Inclusion criteria:** - Patients who have received anti-TNF therapy Etanerecept (Enbrel) or Infliximab from 4-6 months and adults (18 years of age or over). **Exclusion criteria:** - Women who are pregnant or breast-feeding, newly diagnosed patients and Patients with other chronic diseases (ex. Diabetes mellitus and Hypertension).

Study design

SYBR Green-based quantitative real-time polymerase chain reaction (qPCR) allelic specific assay for HLA-G gene (Agilent, USA), which is (rs 1063320) SNP . Genomic DNA had been extracted from whole blood for 200 samples for patients and

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control by using DNA extraction kit from whole blood- MBead Blood/Cell Genomic DNA Kit. The computer programs were used for primer sequencing which was NCBI Blast, NCBI gene and ApE. Table (1)

Table (1) Primers sequences used to detect HLA-G SNP.		
HLA-G	rs1063320	Insertion/forward primers 5'- ACC CCT CAC TGT GAC TGA TAT-3' insertion/revers5'-AGG CAT GAA CAA ATC TTG CCA C-3' Deletion/ forward primers 5'- ACC CCT CAC TGT GAC TGA TAT-3 Deletion/revers 5'-TCA CAA AGG GAC TTG CCA CTC AG-3.

The lyophilized primers were prepared according to the manufacture company information. Real-time PCR was performed in a total volume of 20µL containing 10µL of 2x SYBR Green PCR Master Mix, 1µL of each primer per reaction, 2µL of the genomic DNA dilution, and distilled water to the final volume. The PCR thermal profile was as follows: initial denaturation step (95c° for 3 min, 1 cycle) was followed by amplification and quantification steps repeated for 40 cycles (95c°for 5 sec, primer set annealing temperature for 15sec,72c° for 20 sec, with a single fluorescence measurement at the end of the elongation step at 72c°.

ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to Human HLA-G high resolution melting-curve program analyzed the data. Detection of sHLA-G done according to manufacture procedure (ELIZA reader and washer/ biotek/ USA, kit/ Mybiosource / USA).

3. Statistical analysis

Data analysis was computer assisted using SPSS21 (statistical package for social sciences) and Microsoft excel 2020. Parametric variables are expressed as median and 25-75 confidence interval. Comparison was done using Mann–Whitney *U* test for tow variables while Wilcoxon rank test for three or more groups. Non-parametric variables are expressed as frequency and percentage. The comparison of these variables between study groups was done using Fisher exact test and chi square test. P value less than the 0.05 was considered statistically significant. Odd ratio and confidence intervals were used to assess the risk or beneficial effect of studied gene polymorphisms between groups

4. Results

HLA-G polymorphism in study groups

Human leucocyte antigen 3 prime untranslated region (UTR) Variant or rs1063320 C>G / C>T found on chromosome 6 was present in three genotype CC, CG and GG with two allele C (mutant allele) and G (wild allele). The qReal-Time PCR results in RA patients and controls showed that the frequency of heterozygous (wild/mutant) (CG) genotype was highest in patients group 79(79.0%) compare with 43(43.0%) in control group, while genotype of homozygous(wild/wild) (GG) had lowest frequency in patients 9(9.0%). Further calculation of allelic frequency indicated a frequency of mutant allele (C) in patients group was 103(51.5%). On other hand, the frequency of wild allele (G) was more than (C) allele in the control group (157(78.5%) vs. 97(48.5%)) in patients group with odd ratio or risk to developed RA=3.88. Table (2).

Table (2) HLA-G gene polymorphism in study groups.							
		Patients	Controls	P value	Odd ratio		CI
HLA-G Genotype	CC	12 (12.0%)	0 (0.0%)	<0.001			
	CG	79 (79.0%)	43 (43.0%)				
	GG	9 (9.0%)	57 (57.0%)				
HLA-G allele	C	103(51.5 %)	43 (21.5%)	<0.001	3.88	2.52	6.03
	G	97 (48.5)	157 (78.5%)				
CI : confidence interval.							

Regarding the association between HLA-G gene polymorphism and response to biological drugs which were responders (9 patients) and non-responders (91 patients) the results showed no significant association between **them P=0.9. Table (4.9).**

Table (3) Association between HLA-G polymorphism and response to biological drugs				
		Response		P value
		Responder (N=9)	Non responder (N=91)	
HLA-G genotype	CC (n=12)	1 (8.3%)	11 (91.7%)	0.944
	CG (n=79)	7 (8.9%)	72 (91.1%)	
	GG (n=9)	1 (11.1%)	8 (88.9%)	
HLA-G allele	C	9 (8.7%)	94 (91.3%)	0.544
	G	9 (9.3%)	88 (90.7%)	

Table (3), showed no significant association between (rs1063320) genotypes and disease score activity (DAS28).

Table (4) Association between HLA-G genotyping and DAS28.							
		DAS 28				Total	P value
		Remission	Mild	Moderate	Active		
HLA-G genotype	CC	0 (0.00%)	3 (25.00%)	8 (66.70%)	1 (8.30%)	12 (100.00%)	0.133
	CG	9 (11.40)	15 (19.00%)	38 (48.10%)	17 (21.50%)	79 (100.00%)	
	GG	2 (22.20%)	1 (11.10%)	5 (55.60%)	1 (11.10%)	9 (100.00%)	
Total		11	19	51	19	100	
HLA-G allele	C	9 (8.70%)	21 (20.40%)	54 (52.40%)	19 (18.40%)	103 (100.00%)	0.724
	G	13 (13.40%)	17 (17.50%)	48 (49.50%)	19 (19.60%)	97 (100.00%)	
Total		22 (11.00%)	38 (19.00%)	102 (51.00%)	38 (19.00%)	200 (100.00%)	

Present results revealed no significant association of HLA-G 14 bp ins/del variant with sex, smoking, RF and anti-CCP, but there was a significant association with age groups. Table (4).

Table (5) Age groups, sex, smoking, RF and anti-CCP in association with HLA-G genotyping						
		HLA-G genotype			HLA-G allele	
		CC	CG	GG	C	G
Age groups	< 30 years	0 (0.00%)	22 (100.00%)	0 (0.00%)	11 (50.00%)	11 (50.00%)
	30-39 years	6 (9.40%)	44 (68.80%)	14 (21.90%)	28(43.80%)	36 (56.20%)
	40-49 years	2 (4.00%)	46 (92.00%)	2 (4.00%)	25 (50.00%)	25 (50.00%)
	50-59 years	10 (23.80%)	32 (76.20%)	0 (0.00%)	26 (61.90%)	16 (38.10%)
	> 60 years	6 (27.30%)	14 (63.60%)	2 (9.10%)	13 (59.10%)	9 (40.90%)
P value		<0.001			0.415	
Sex	Females	24 (14.10%)	130 (76.50%)	16 (9.40%)	89 (52.40%)	81 (47.60%)
	Males	0 (0.00%)	28 (93.30%)	2 (6.70%)	14 (46.70%)	16 (53.30%)
P value		0.068			0.566	
Smoking	smokers	2 (4.20%)	42 (87.50%)	4 (8.30%)	23 (47.90%)	25 (52.10%)
	nonsmokers	22 (14.50%)	116 (76.30%)	14 (9.20%)	80 (52.60%)	72 (47.40%)
P value		0.147			0.569	
RF	Negative	10 (11.40%)	70 (79.50%)	8 (9.10%)	45 (51.10%)	43 (48.90%)
	Positive	14 (12.50%)	88 (78.60%)	10 (8.90%)	58 (51.80%)	54 (48.20%)
P value		0.970			0.927	
Anti-CCP	Negative	4(6.20%)	56 (87.50%)	4(6.20%)	32 (50.00%)	32 (50.00%)
	Positive	20 (14.7%)	102 (75.00%)	14 (10.30%)	71 (52.20%)	65 (47.80%)
P value		0.120			0.771	

In addition, the current study revealed that the soluble HLA-G (sHLA-G) level in RA group was lower than that in healthy control group but statistically not significant. Table (5).

Table (6) HLA-G serum level in patients and control groups.			
		Groups	
		Patients	Controls
HLA-G Concentration	Median	0.24	0.30
	Percentile 25	0.14	0.16
	Percentile 75	1.00	1.95
P value		0.121	

Furthermore, Table (6) show significant relationship between serum level of sHLA-G and DAS28 p=0.01.

Table (7) Association between sHLA-G and DAS28 in patients group.				
sHLA-G	DAS score			
	Remission	Mild	Moderate	Active
Median	0.15	0.24	0.28	0.18
Percentile 25	0.11	0.16	0.14	0.13
Percentile 75	0.30	3.80	1.10	0.30
P value	0.010			

Concerning sHLA-G concentrations there was no significant differences among the three HLA-G genotypes with sHLA-G concentrations,P value= 0.08 Table (7).

Table (8) HLA-G genotyping and serum level of sHLA-G in RA group.					
sHLA-G	HLA-G gynotyping			HLA-G alleles	
	CC	CG	GG	C	G
Median	0.19	0.25	0.28	0.23	0.26
Percentile 25	0.11	0.14	0.16	0.14	0.14
Percentile 75	0.30	1.80	0.30	1.00	1.00
P value	0.089			0.548	

5. Discussion

Current study showed that the HLA-G rs1063320 genotype increased risk of RA and this result confirmed an association of HLA-G polymorphism with the susceptibility to RA. This finding agrees with Catamo, *et al* thier results indicate a possible association between HLA-G gene polymorphisms and susceptibility to develop RA disease and its severity (10). Other study showed that the functional HLA-G SNP polymorphisms are associated with susceptibility to systemic lupus erythematosus (SLE) and RA (11). HLA-G polymorphism in exon 8 in the 3'UTR of HLA-G was found to be associated with the stability and splicing patterns of HLA-G mRNA isoforms (12).

Study published in 2007 found that the presence of a guanine at the +3142 position (rs1063320) SNP may influence the expression of the HLA-G locus by increasing the affinity of this region for the **miRNAs:** miR-148a, miR-148b and miR-152; therefore, decreasing the mRNA availability by mRNA degradation and translation suppression (13). Lee, *et al* showed that the HLA-G 14-bp I/D polymorphism is associated with susceptibility to a subgroup of autoimmune diseases such as SLE, but not RA (14). Other studies did not support an association between HLA-G 14 bp ins/del variant and risk/protection of RA (15, 16, 17).

In the current study, rs1063320 SNP in HLA-G gene had not a role in response to Etanarecebet (Enbrel) or Infliximab. In this field, other study have indicated the association of polymorphisms with several clinical situations, including modulation of the therapeutic response, it is prudent to emphasize that these results are still controversial and more investigation is required to prove its potential properties (18). .

As well as in this study the HLA-G polymorphism was associated with age but not with sex, smoking, RF or Anti-CCP status. Other study showed that the HLA-G does not appear to be a risk factor for development of RA in south Indian but may act as a genetic modifier of clinical phenotype in terms of autoantibody production, gender preference and age at disease onset (19).

This work exhibited no relationship between HLA-G genotype and disease activity score. Study by Rizzo R, *et al* found that the frequency of 14 bp deletion allele increased in patients with disease remission. Based on these results, HLA-G may be a candidate biomarker to evaluate early prognosis and disease activity in RA patients (20).Other study reveal that no significant association was found between the polymorphism and early disease activity (21).

Concerning, soluble HLA-G (sHLA-G) present study showed that the sHLA-G level was lower in patients than control group but statistically not significant. Moreover, in this research was no association between sHLA-G and HLA-G genotype. Study by Veit , *et al* showed that the presence of the G allele in homozygosis could be responsible for a low HLA-G expression profile that could favor the triggering of RA (22). Verbruggen, *et al* found that the sHLA-G level in RA patients were significantly lower than healthy controls. They suggested that patients with low sHLA-G levels were unable to suppress self-reactive cells leading to development of autoimmunity. Soluble HLA-G strongly inhibits T and natural killer (NK) cell functions, low sHLA-G suggests that T and NK cell activities are not efficiently restricted by sHLA-G molecules in rheumatoid arthritis (23). Previous report had association between the presence of the 14bp insertion allele (+14bp) to an unstable mRNA and a lower sHLA-G protein production, suggesting a different ability to counteract inflammation between genotypes (24).

It is worthy to mention that the sHLA-G was associated with DAS28. This result similar to other

study which showed that the sHLA-G expression inversely correlated with DAS28 disease scores (25). However, other study showed no noticeable correlation between plasma sHLA-G levels and disease activity parameters (26).

The contrasting results obtained by Manaster and coauthors who have reported the absence of +3142C>G effect on the miRNA control of membrane HLA-G expression, prompt further considerations on the relationship between this polymorphism and membrane HLA-G expression (27). In the current study the using of biological drugs; also the small sample size may be effect on serum level of sHLA-G.

6. Ethics Committee Approval

Ethical approval and informed consent were obtained from each participant in this study according to the declaration of Helsinki-ethical agreement, it was obtained from the Institutional Review Board of College of Medicine /Al- Nahrain University (I.R.B/ 186 30/1/2019).

7. Conflict of interest

The author declares no conflict of interest.

8. Funding

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