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Original paper

Association of HLA-G polymorphism and susceptibility to toxoplasmosis infection in rheumatoid arthritis patients

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ABSTRACT. *Toxoplasma gondii* (*T. gondii*) is an intracellular protozoan parasite that can infect approximately one-third of the world's human population. HLA-G level change is one immune evasion tactic in the host-parasite interaction. The immune system can be suppressed by HLA-G, a special protein (non-classical HLA class I) molecule that has the ability to control natural killer cell (NK) activity such as cytotoxicity and cytokine production through NK cell receptors. HLA-G level modification is one immune evasion mechanism in the host-parasite interaction. The immune system can be suppressed by HLA-G, a special protein (non-classical HLA class I) molecule that has the ability to control natural killer cell (NK) activity such as cytotoxicity and cytokine generation through NK cell receptors. This study aimed to investigate the alteration in sHLA-G levels could be impressed by present of *Toxoplasma* and rheumatoid as well as the seroprevalence of *Toxoplasma gondii* (*T. gondii*) antibodies in Iraqi patients with rheumatoid arthritis was investigated. The prevalence of anti-*T. gondii* IgG was 50% in arthritic patients in comparison to 41.6% in healthy controls. No positive anti-*T. gondii* IgM was detected. In the current study evaluate the possible association of the HLA-G gene polymorphisms with susceptibility to RA and toxoplasmosis by study two polymorphism at exon 8 (rs17179101 and 14 bp ins/del). Ultimately there were significant association between two diseases that have been studied and 14 bp ins/del and snp (rs17179101).

Keywords: rheumatoid, *Toxoplasma*, sHLA-G

Introduction

Toxoplasmosis is a zoonotic infection caused by the obligatory intracellular apicomplexan parasite *Toxoplasma gondii*. Swallowing undercooked or raw meat from infected animals, or consuming food or water contaminated with oocysts shed by infected felids, can result in postnatal *T. gondii* infection [1]. Felids are the only definitive hosts of *T. gondii*, and they play an important role in the parasite's transmission [2]. Although this parasite usually has no adverse effects in immunocompetent individuals, it is now recognized that the condition may be a risk factor for a number of immune system diseases, including rheumatoid arthritis (RA) [3].

Rheumatoid arthritis is a chronic inflammatory disease of unknown etiology that typically affects synovial joints but is also characterized by a broad range of extra-articular symptoms [4]. The clinical course of RA varies from mild to severe disease, which can result in joint damage, chronic disability, and early mortality [5].

Autoimmunity related with the disease, such as anti-citrullinated protein antigens (ACPAs), are produced well before the first clinical symptoms of rheumatoid arthritis [6]. Induced citrullination of proteins can consequently cause a breach of peripheral immune tolerance to self-antigens, leading to inflammation and autoimmunity [7]. Opportunistic infection with *T. gondii* is an increasing problem in association with inflammatory rheumatoid [8].

There is growing interest in exploring the link between infection with this parasite and autoimmune diseases, given the propensity of *T. gondii* infection to occur in immunocompromised patients [9].

Geraghty et al. [10] were the first to identify the

Table 1. Components of reaction mixture for amplification of HLA-G

Component	Volume (µl)
Master mix EasyTaq® PCR SuperMix	12.5
Forward primer	1
Revers primer	1
DNA template	3
Nuclease-free water	7.5
Total	25

Human Leukocyte Antigen-G gene as a member of the non-classical Class I. Class I molecules are heterodimers composed of a heavy chain and a light chain (beta-2 microglobulin). The HLA-G can bind receptors on several immune cell types and inhibit the function or induce apoptosis of CD8 natural killer and T cells [11]. HLA-G-positive antigen-presenting cells are also potent inhibitors of CD4 T cell proliferation. Therefore, a role of HLA-G could be expected in autoimmune diseases and in particular in rheumatoid arthritis [12].

The HLA-G gene is located within the major histocompatibility complex on the chromosome 6, contains a 14 bp insertion (ins)/deletion (del) and a ((rs17179101) polymorphism in 3′-untranslated region (3′UTR) of HLA-G. Some previous studies reveal the relationship of RA and HLA-G 14 bp ins/del. Rizzo et al. [13] found that the frequency of 14 bp del allele was associated with RA remission and concluded that HLA-G may be a candidate biomarker to evaluate early prognosis and disease activity in ERA patients. On the other hand, Lee et al. [14] revealed no significant association between HLA-G 14 bp ins/del and RA risk. Because RA is an inflammatory disease with autoimmune aspects, and the HLA-G molecule has anti-inflammatory properties via developing immunological tolerance, there were no previous research study association between HLA-G 14 bp ins/del and *Toxoplasma* infection.

In this study as a result, we decided to investigate the potential link between HLA-G gene polymorphisms with RA and toxoplasmosis susceptibility.

Materials and Methods

Sample collection

A total of 150 blood samples were collected from women with rheumatoid arthritis (RA) attends the Rheumatology Department in Baghdad Teaching Hospital in Baghdad governorate. Out of those 150 RA patients only 50 patients were including in this study. This selection was done according to exclusion of any patient had negative RF or CRP or ACCP test for homogeneity. Blood samples were also collected from 60 apparently healthy women. All patients and healthy control women were subjected to IgM and IgG Toxo ELISA kit (Foresight-UAS) to investigate any toxoplasmosis infection. Women without toxoplasmosis were considered a negative control group while women with toxoplasmosis were considered a positive control group. All the samples succumbed to diagnostic tests with HLA-G (Bioassay Technology Laboratory-China).

Table 2. PCR program conditions for amplification of HLA-G

Thermal cycler protocol	Temperature (ºC)	Time (min:sec)	Cycle
Initial denaturation	95	3:00	1
Denaturation	95	0:30	
Annealing	60	0:30	
Extension	72	1:00	30
Final extension	72	5:00	1

Table 3. The percentage distribution of RA patients and healthy donors according to Toxo ELISA test IgM and IgG tests (NS=non significant)

Test subject	Total	Toxo IgM acute		Toxo IgG chronic		Negative samples	
		No	(%)	No	(%)	No	(%)
RA patient	50	0	0	25	50	25	50
Healthy control	60	0	0	25	41.6	35	58.33
Chi-square	–	–	NS	–	2.603 NS	–	2.557 NS

Table 4. The levels of sHLA-G in studied samples

Group		No	Mean ± SE HLA-G (pg/ml)
RA patients (n=25)	Treated	15	10.72±1.35a
	Untreated	10	6.37± 0.54 bc
Toxoplasmosis+RA (n=25)	Treated	15	7.72± 0.64 b
	Untreated	10	4.09± 0.61cd
Toxoplasmosis (n=25)		25	6.68± 0.78 bc
Control (n=35)		35	3.79± 0.31 d
LSD value		–	2.072 **
P-value		–	0.0038

Explanations: different letters in columns indecte a significantly difference;** ($P \leq 0.01$)

Polymerase chain reaction amplification of the rs17179101 and 14 bp polymorphism in exon 8 (3 UTR) of the HLA-G gene and genotyping

DNA was extracted from 200 µl of serum samples using the the EasyPure® Blood Genomic DNA Kit (Transgen, China) according to the manufacturer's instructions. Then, PCR was used to amplify the HLA-G region containing the polymorphism, forward 5′-GTGATGGGCTGTTTAAAGTGTCACC-3′ and revers 5′-GGAAGGAATGCAGTTCAGCA TGA-3′ primers [15]. The PCR components of reaction mixture for amplification of HLA-G shows table 1. Amplification of DNA was achieved according to the condition (Tab. 2).

Amplification products for the two SNPs of HLA-G gene (rs17179101 and 14 bp) were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation, Korea. Then sequences were compared with reference sequences of HLA-G gene information in Genbank of the National Center for Biotechnology Information (NCBI). Data was analyzed using genious software.

Statistical analysis

The Statistical Analysis System – SAS, 2018 program was used to detect the effect of difference factors in study Darameters. Least Significant Difference – LSD test (Analysis of Variation – ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01) probability. Also, we estimated of odd ratio and CI.

Results

All the patients samples were subjected to Toxo ELISA tests (IgM and IgG), the result elucidated that only 25 RA patients were infected with chronic toxoplasmosis (RA +ve Toxo +ve IgG), as well as 25 apparently healthy donor were also with chronic toxoplasmosis while the other 35 were healthy (control negative) (Tab. 3).

In relation to other results, the current results showed that treated RA patients without coinfection with *Toxoplasma* had the highest significant (10.72±1.35 pg/ml) increase in the level of sHLA-G in comparison to RA untreated patients (6.37±0.54 pg/ml).

However, treated RA co-infected patients with *Toxoplasma* showed high significant increase of sHLA-G (7.72±0.64 pg/ml) in comparison to co infected untreated (4.09±0.61 pg/ml) (Tab. 4). Additionally, when looking to the result of sHLA-G level in patients with

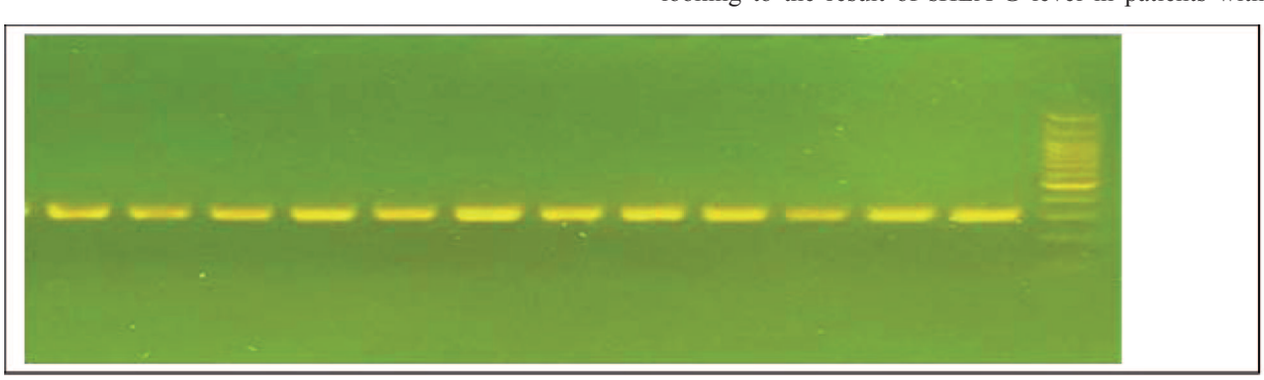

Figure 1. Amplified products of exon 8 of HLA-G gene after electrophoresis on 1% agarose gel at 70 v/cm for 75 min

Table 5. Distribution of sample study according to *HLA-G exon 8* polymorphism rs17179101 in control and patients groups

HLA-G exon 8 polymorphism rs17179101	Frequencies (%) Control group (n=25)(%)	Patients group (n=50)(%)			*P*-value	Odd ratio (95% CI)
		RA	TOXO	RA+TOXO		
CC	(n=25)(100)	15(60)	6(60)	8(53.3)	0.503 NS	Ref.=1
CA	(n=0)(0)	6(24)	3(30)	7(46.6)	0.021*	0.778(0.305–1.16)
AA	(n=0)(0)	4 (16)	1(10)	0(0)	0.0361*	1.02 (0.67–1.74)
Allele frequency						
C	1		0.74			–
A	0		0.26			–

Explanations: * $P \leq 0.05$; NS: non significant

toxoplasmosis only, the result obtained that its level was significantly ($P \leq 0.01$) increased (6.68±0.78 pg/ml) in comparison to control group (3.79±0.31 pg/ml). In exon 8 of HLA-G gene, two polymorphisms were examined by amplification the sites of polymorphisms (rs17179101 C/A and 14 bp ins/del).

Results of amplification was showed in figure 1. A sharp band with molecular size of 244 bp was detected after electrophoresis on agarose gel 1% in present of DNA ladder marker.

Results indicated in table 5, also showed that the CC genotype what the predominant (100%) in healthy control women examined for rs17179101 in exon 8, while this homozygous non-polymorphic was 60, 60 and 53.3% in RA, toxoplasmosis women, and RA with co-infection toxoplasmosis women respectively, at the time that CA was the most common genotype in all patients groups, while homozygous polymorphic genotype AA was the lowest among all groups as indicated in tables 1 to 4.

The CC homozygous genotype was not differ significantly among the studied groups, while CA heterozygous polymorphic, and the AA homozygous polymorphic genotypes were significantly different ($P<0.01$) between each patients group (RA patients, toxoplasmosis patients and RA-toxoplasmosis patients).

Furthermore odd ratio for CA and AA genotypes in the studied groups, were 0.7 and 1.0 respectively, which motioned that C>A polymorphism was protective but not a risk factor for incidence of RA, toxoplasmosis and RA associated toxoplasmosis.

Another polymorphism studied in present study was HLA-G (14 bp ins/del). Results indicated in table 6, showed that the del/del genotype (50%) in healthy control women, while this homozygous was 23.07, 0 and 16.66% in RA, toxoplasmosis women, and RA with co-infection toxoplasmosis women, respectively.

Furthermore, the genotype 14 bp ins/del was 12.5% in control group, while 46.16, 40 and 12.5% in RA,

Table 6. Genotype distribution and allele frequencies of HLA-G 14-bp ins/del gene polymorphism in patients and controls

Genotype	Patients (n=24)			Controls (n=8) No (%)	*P*-value	OR (CI 95%)
	RA No (%)	TOXO No (%)	RA+TOXO No (%)			
del/del	3(23.07)	0(0)	1(16.66)	3(37.5)	0.0001 **	Ref.=1
ins/del	6(46.16)	2(40)	3(50)	1(12.5)	0.0006 **	1.63 (0.86–3.47)
ins/ins	4(30.77)	3(60)	2(33.34)	4(50)	0.0097 **	1.44 (0.79–2.75)
Allele frequency						
del		19(39.58)		7(43.75)	–	–
ins		29(60.42)		9(56.25)	–	–

** $P \leq 0.01$

toxoplasmosis women, and RA with co-infection toxoplasmosis women, respectively.

The homozygous genotype ins/ins was 37.5% in control, while the 30.77, 60 and 33.34% in RA, toxoplasmosis women, and RA with co-infection toxoplasmosis women, respectively.

Discussion

A heightened risk of *T. gondii* infection in patients with rheumatic diseases can be attributed to alterations in innate and adaptive immune responses. Patients with RA were found to be highly susceptible to *T. gondii* infection – particularly during periods of immunosuppression that followed treatment. So, any reduction in the body's defenses against infection places arthritis patients at risk.

Patients with rheumatoid arthritis have an aberrant regulatory network for the immune response, including the HLA-G [16]. The present study confirmed earlier findings by revealing that blood sHLA-G protein concentrations were considerably lower in RA patients who were not receiving treatment. which agreed with previous findings by Rizzo et al. [13] and Shakir and Al-Qadhi [17]. Reduced sHLA-G concentrations may cause persistent inflammatory cell activation and contribute to disease progression [17].

Chronic synovial inflammation in rheumatoid arthritis (RA) damages articular cartilage and bone over time, eventually leading to disability. For RA patients, a number of disease-modifying anti-rheumatic drugs (DMARDs) are available. Conventional synthetic DMARDs, particularly biological DMARDs, have been demonstrated to effectively inhibit joint destruction in RA. DMARDs include many drugs, such as methotrexate, hydroxychloroquine, sulfasalazine, and leflunomide. The DMARD therapy has ability to modify HLA-G secretion by enhances HLA-G secretion by peripheral blood mononuclear cells [19]. As a result, drugs used to treat RA cause an increase in HLA-G levels and increasing the risk of toxoplasmosis [18]. As mentioned, HLA-G antigens are characterized by anti-inflammatory and immuno-inhibitory functions, thus the presence of these molecules could affect disease activity [19].

Rizzo et al. [13] show that the chronic activation of inflammatory cells and contribute to the development of autoimmune diseases, and the current study found that when the amount of sHLA-G was low, there was a severe infection with one of the autoimmune diseases (RA).

Toxoplasma infection causes NK cells to activate, and sHLA-G has been shown to maintain immunologic tolerance by interacting with dNK inhibitory receptors to inhibit the cytotoxicity of NK cells, which results in lower sHLA-G levels in RA-coinfected patients compared to RA patients without *Toxoplasma* infection.

More recently, in another cohort, in Iraq by Shakir and Al-Qadhi [17] also agreed with the current study result when they mentioned that the sHLA-G level in toxoplasmosis group and Toxo-coinfection with RA was higher than its level in control group.

Studied exon 8 SNPs (14 bp ins/del and rs17179101) are belong to HLA-G 3′UTR and the nucleotide sequence of HLA-G 3′UTR was first described by Geraghty [10]. Encompassing approximately 1000 nucleotides and presenting several variations sites, some of them are associated with distinct expression profiles [20]. Since the magnitude of HLA-G expression is regulated in part by the gene promoter and 3′ untranslated region (3′UTR), and since a differential expression of HLA-G has been reported even for healthy individuals [20]. As well as, the HLA-G 3`untranslated region (UTR) contains regulatory elements included polyadenylation signals and AU-rich sequences [21], which play important role in the spatial and temporal expression of HLA-G [22]. Many studies have focused on the study of variable sites at the regulatory segments [23]. In the present study, we investigated the impact of 14 bp ins/del polymorphism on risk of RA and toxoplasmosis in a sample of Iraqi population, allele and genotype frequencies of HLA-G 14-bp ins/del polymorphism were analyzed in studied and controls groups. As in other world populations, both alleles (ins and del) showed polymorphic frequencies in the Iraqi sample of controls (56.25 and 43.75%, respectively). In many populations ins and del were investigated. Ins was the allele of minor frequency and had a range of 23.6% in Koreans [24] to 49.2% in Saudi Arabians [25], however, Egyptians [26] and Tunisians [27], showed the opposite profile, and the Ins allele frequency exceeded this range (60 and 51.2%, respectively). Therefore, the Iraqi Ins allele frequency fits well the presented range.

Some previous studies reveal the relationship of RA and HLA-G 14 bp ins/del, Rizzo et al. [13] found that the frequency of 14 bp del allele was associated with RA remission and concluded that HLA-G may be a candidate biomarker to evaluate early prognosis and disease activity in ERA patients. On the other hand, Lee et al. [14] revealed no significant association between HLA-G 14 bp ins/del and RA risk.

Veit et al. [28] also have observed no differences in allelic and genotypic frequencies of the HLA-G 14 bp ins/del polymorphism between RA patients and controls, the comparison between patients groups and controls depicted variations in allele and genotype frequencies of HLA-G 14 bp ins/del polymorphism.

Another study was in south Indian by Mariaselvam et al. [29] deal with polymorphisms in 3′UTR (which include14 bp ins/del) they referred there is no association between polymorphisms of HLA-G and risk of RA development.

Several studies published in the literature have established therapy response and illness risk. Several studies have suggested that the folate pathway plays an important role in the clinical effects of MTX treatment in

RA, related to genetic differences in the methylene tetrahydrofolate reductase (MTHFR) gene. Baricordi et al. [30] discovered a link between the HLA-G 3UTR 14 bp ins/del polymorphism and clinical response to MTX treatment in RA, and a high frequency of HLA-G 14 bp ins/ins genotype was found in MTX responder patients. The meta-analysis performed by Lee et al. [31] did not show any linkage between the HLA-G 14 bp ins/del polymorphism, MTX response, and the risk of RA. Furthermore, Rizzo et al. [32] reported that MTX responders were more likely to have 14 bp del/del genotype as compared to non-responders as this genotype results in higher expression of sHLA-G. In contrast, similar studies have found no effect of the HLA-G 14 bp ins/del polymorphism on treatment response or disease risk [33].

Verbruggen et al. [34] found that the levels of sHLA-G in patients with RA 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. The 3′-untranslated region (UTR) has a major role in HLA-G regulation, It has been proposed that polymorphism exerts a significant effect in the HLA-G function and may have an impact on the expression of sHLA-G [35]. The discrepancy in findings among studies may be due to genetic and environmental differences between the different populations being investigated. The 14 bp ins/del has been studied with many parasite like *T. gondii* showed no significant difference between toxoplasmosis patients and healthy control in the heterozygous and homozygous of 14 bp gene polymorphism [36].

In summary, we found a significant association between HLA-G 14 bp ins/del and susceptibility to RA and *Toxoplasma* in a sample of Iraqi women. This research provided the role of HLA-G's activity in both of toxoplasmosis and rheumatoid arthritis patients as well as show the association between HLA-G 14 bp ins/del and susceptibility to RA and *Toxoplasma.* Further association studies with large sample size and different ethnicities are required to verify our findings.

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