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ORIGINAL ARTICLE

Association of Human Leukocyte Antigen-G Gene Polymorphisms with Rheumatoid Arthritis: Relationship with Disease Activity, Severity and Treatment Response

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ABSTRACT

Background: Human leukocyte antigen (HLA-G) plays a role in inflammation and autoimmune disorders. Variations in the HLA-G gene may have an effect on rheumatoid arthritis (RA). This study examined the impact of the polymorphisms +3142G>C and 14 bp ins/del on disease susceptibility, activity, severity, and responsiveness to therapy in a cohort of Egyptian RA patients.

Methods: This case-control study was done on 75 patients with RA and 75 healthy people. The HLA-G rs1063320 (+3142G>C) and rs66554220 14 bp insertion (ins)/deletion (del) variants were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFP) and PCR technique, respectively.

Results: Our finding didn't support an association between HLA-G 14 bp ins/del or the HLA-G +3142G>C variants and RA susceptibility. Subcutaneous nodules and prolonged morning stiffness were substantially related with the G allele of HLA-G +3142G>C. We used the disease activity score 28 (DAS-28) to look for a correlation between the HLA-G gene variations and disease activity, but we were unable to find one. Higher levels of C-reactive protein (CRP) and autoantibodies (anti-citrullinated protein antibodies (ACPA) and ACPA+ rheumatoid factor (RF)) have been linked to the GG genotype and the G allele of HLA-G +3142G>C. Higher rheumatoid arthritis severity scale (RASS) was found to be related to the HLA-G+3142G>C polymorphism and RA severity. The 14 bp ins/del polymorphism and treatment response were found to be significantly correlated. Patients with the del/del genotype and the del allele had a significantly higher percentage of satisfactory response to therapy.

Conclusion: The 14 bp ins/del and the +3142G>C of HLA-G gene didn't appear to be associated with RA susceptibility in this studied group of RA patients. But in terms of autoantibody production, severity, clinical phenotype, and treatment responsiveness, it may operate as a genetic modulator of disease phenotype. In this regard, we propose that rather than serving as a risk factor for the onset of RA, these polymorphisms serve as disease modifiers. Treatment response and polymorphism were discovered. Patients with the del/del genotype and the del allele had a significantly higher percentage of satisfactory response to therapy.

Keywords: Egyptian, genetic, polymorphisms, human leukocyte antigen-G, rheumatoid arthritis.

INTRODUCTION

The most prevalent autoimmune rheumatic illness with an uncertain etiology is rheumatoid arthritis (RA) [1]. In Egypt, the estimated prevalence of RA is between 0.09% and 0.10%, with a higher incidence of the disease in women (5.4:1) and a younger age of disease beginning than in other nations [2-4].

It has been suggested that the manifestation of the disease and its complications are influenced by both genetic and environmental variables. Up to 60% of the risk of RA is thought to be influenced by genetic factors [5].

Human leucocyte antigen-G (HLA-G), a nonclassical major HLA class Ib protein, may decrease the activity of natural killer (NK) cells, CD4+, CD8+ lymphocytes, and dendritic cells [6,7].

The HLA-G gene, which is located on chromosome 6 (6p21.31), has a 14 bp ins/del and a +3142G>C (rs1063320) polymorphism in its 3′-untranslated region (3′UTR). Plasma HLA-G expression levels and rates are impacted by 3′UTR polymorphism [8,9].

The 14bp ins/del polymorphism in exon 8 of the 3′UTR of HLA-G was found to have an impact on the stability and splicing patterns of HLA-G messenger ribonucleic acid (mRNA) isoforms. When compared to the homozygous insertion genotype, the homozygous 14 bp deletion genotype produces a more stable mRNA [10,11]. The ins allele is linked to low levels of serum HLA-G (sHLA-G) [12].

The affinities of different microRNAs that inhibit HLA-G synthesis on HLA-G mRNA targets are impacted by the +3142G>C polymorphism [11]. RA patients' plasma soluble sHLA-G levels were found to be lower than those of controls [10].

Higher levels of sHLA-G and a higher frequency of the 14 bp deletion allele, which has been suggested as a potential prognostic marker in RA, were found in the blood of patients who reacted to anti-RA treatment [13,14].

Using a sample of RA patients from Egypt, the current study aimed to ascertain whether the HLA-G gene polymorphisms rs1063320 (+3142G>C) and rs66554220 (14 bp ins/del) are connected to RA disease susceptibility, activity, severity, and therapy response.

METHODS

The trial included 150 participants, including 75 RA patients who fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism for RA [15] criteria and 75 control individuals.

The cases were selected from RA patients attending the Rheumatology and Rehabilitation Department, Faculty of Medicine, Zagazig University Hospitals. Patients were symptomatic for ≤3 years and were drug naïve newly diagnosed cases or previously diagnosed RA cases without treatment for at least 6 months. Cases with other autoimmune diseases or comorbidities were excluded.

Patients were excluded if they had a history of cancer, a chronic inflammatory condition, an infection of any kind, diabetes mellitus, uncontrolled hypertension, any type of cardiac disease, renal failure, or liver dysfunction. Also were excluded if they had good results with CPAP. Female gender (hormonal effect hematological disease) and BMI > 30kg/m2 were also excluded.

Seventy-five individuals in good health who were age- and sex-matched to RA patients, had no known autoimmune disorders, no family history of the condition, and were from the same region as the RA patients made up the control group.

The study was approved by Institutional Review Board (IRB) at Faculty of Medicine, Zagazig University (ZU-IRB#2253) and informed consent was obtained from all participants. Written informed consent was obtained from all participants. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

For the subsequent follow-up research, cases were included. All participants in the trial underwent initial evaluations (pre-treatment), after which they received the standard of care for RA (conventional synthetic disease-modifying anti-rheumatic medications). At the

start and the end of the follow-up study (after 6 months), all patients underwent tests for CRP, ESR, disease activity score 28, rheumatoid arthritis severity scale, and health assessment questionnaire (HAQ).

At the end of the study patients were reevaluated for response to treatment according to EULAR response criteria by the DAS 28 [16].

HLA-G gene genotyping for subjects in the two groups

Genomic DNA was extracted from peripheral blood samples using the salting-out method and the TIAN genomic DNA kit from TIANGEN (Beijing, China). The HLA-G rs66554220 (14 bp ins/del) mutation was then genotyped using polymerase chain reaction [17,18]. The forward and reverse primers were 5′-TCACCCCTCACTGTGACTGATA-3′ and 5′-GCACAAAGAGGAGTCAGGGTT-3′, respectively [18]. Each 0.20 mL PCR reaction tube included 1 L of genomic DNA (around 100 ng/mL), 1 L of each primer (10 M), 10 L of 2x Prime Taq Premix (Genet Bio, Korea), and 5 L ddH2O.

The PCR cycling conditions were as follows: a first step lasting 5 minutes at 95 degrees, then 30 cycles lasting 95 degrees for 30 seconds, 56 degrees for annealing, and 72 degrees for extension, with a final extension lasting 5 minutes at 72 degrees. The PCR products were separated using 2% agarose gel electrophoresis under UV light. The ins allele's product sizes were 141 bp, while the del allele's were 127 bp. The HLA-G rs1063320 (+3142G>C) variant was genotyped using PCR-RFLP methods. The forward and reverse primers were identified by their respective labels of 5′-CATGCTGAACTGCATTCCTTCC and 5′-CTGGTGGGACAAGGTTCTACTG. [19].

Using a denaturation step at 95 °C for 5 min, 30 cycles of 30 s each at 95 °C, 65 °C, and 72 °C, and a final step at 72 °C for 10 min, amplification was completed. The PCR products in 0 L were digested using the restriction enzyme Fast Digest BseSl. The G allele digested and produced 316 bp and 90 bp, but the C allele produced 406 bp after going undigested.

Statistical analysis:

Using IBM Corp. Released 2015, all data were gathered, tabulated, and statistically analyzed. Version 23.0 of IBM SPSS Statistics for Windows. According to IBM Corp., New York, P value was set at 0.05 for significant results and 0.001 for highly significant results.

RESULTS

This study included 75 RA patients and 75 apparently healthy control subjects (57 females and 18 males; mean age 36.5 7.7 years) and 66 females and 9 males (mean age 38.7 6.2 years), respectively. Age and sex did not significantly differ between the two groups (p = 0.056 and p = 0.09, respectively).

HLA-G rs66554220 (14 bp ins/del) and HLA-G rs1063320 (+3142G>C) polymorphisms were not associated with an increased risk of RA, according to the genotypes, allele frequencies, combination analysis, and haplotype analysis of RA patients and controls (Figure 1).

The G allele of HLA-G rs1063320 (+3142G>C) was substantially linked with the existence of subcutaneous nodules (p=0.024) and a longer duration of morning stiffness (p=0.013) after investigation of the impact of these polymorphisms on the clinical phenotype of RA.

Our findings showed no link between the HLA-G rs66554220 (14 bp ins/del) and rs1063320 (+3142G>C) polymorphisms and DAS-28 disease activity (Table 1, 2).

Patients with the HLA-G+3142G>C GG, GC, and G alleles were considerably more likely to have abnormal CRP levels that were greater than usual (Figure 2).

Regarding the correlation between HLA-G polymorphisms and RA severity measures, individuals with the GG genotype and G allele had significantly higher RASS than those with the CC genotype and C allele (p 0.05) (Table 2).

Only HLA-G rs1063320 (+3142G>C) variant exhibited a connection when the impact of these polymorphisms on the autoantibody

phenotype of RA was examined. We discovered that in the same patient, increased seropositivity for both RF and ACPA was related with the G allele and GG genotype (Table 3, 4).

There is no significant difference between HLA-G rs66554220 (14 bp ins/del) genotyping or alleles and clinical characteristics, parameters of severity and functional status in rheumatoid arthritis patients (p>0.05).

As regards response to treatment, there was significant more percent of good response to treatment among patients with (del/del and ins/del) genotypes, and del allele compared to patients with ins/ins genotype and ins allele respectively (p<0.05) (Figure 3).

Table 1: Frequency distribution of HLA-G rs66554220 (14 bp ins/del) and HLA-G rs1063320 (+3142G>C) polymorphisms in rheumatoid arthritis patients regarding disease activity (According to DAS-28 disease activity score).

Genetics		High activity n.52	Moderate activity n.23	χ^2	p	Odds	95%CI	
							Upper	lower
HLA-G rs66554220 (14 bp ins/del) (gene)	**Gene frequency n (%)**			0.69	0.71			
	Ins/ins	11 (21.2)	3 (13.0)	F	0.68	1.83	0.346	9.72
	Ins/del	31 (59.6)	15 (65.2)	F	0.99	1.03	0.300	3.56
	Del/del	10 (19.2)	5 (21.7)			reference		
	Allele frequency n (%)							
	Ins	53 (51.0)	21 (45.7)	0.36	0.55	1.24	0.617	2.48
	Del	51 (49.0)	25 (54.3)				reference	
HLA-G rs1063320 (+3142G>C) (gene)	**Gene frequency n (%)**			2.4	0.3			
	GG	34 (65.4)	16 (69.6)	F	0.31	1.14	0.0.34	3.4
	GC	13 (35)	7 (30.4)	F	0.27		reference	
	CC	5 (9.6)	0 (0.0)			-		
	Allele frequency n (%)							
	G	81 (77.9)	39 (84.8)	0.95	0.33	0.63	0.250	1.59
	C	23 (22.1)	7 (15.2)				reference	

χ 2: Chi square test of significant, F: Fisher Exact test, p>0.05: non-significant,
CI: confidence interval, n: number

Table 2: Relation of HLA-G rs1063320 (+3142G>C) genotyping and alleles with parameters of disease activity and severity in rheumatoid arthritis patients.

Variables		Rheumatoid arthritis HLA-G rs1063320 (+3142G>C) genotyping			χ^2	p
		GG n.50	GC n.20	CC n.5		
DAS28	Mean± SD	5.98±0.87	6.1±0.99	6.4±0.29	0.44	0.645
HAND X RAY (SES)	Median(range)	5.98±0.87	6.1±0.99	6.4±0.29	0.438	0.647
HAQ	Median(range)	6±1.6	5.5±1.6	5.9±1.4	0.75	0.48
RASS	Mean± SD	55.2±9.2	52.4±10.7	34.9±3.4	10.6	**0.0001**
Variables		**Rheumatoid arthritis HLA-G rs1063320 (+3142G>C) allele**			χ^2	**p**
		G allele n.120	**C allele n.30**			
DAS28	Median(range)	7.5(2-18)	7(3-16)		0.000	1
HAND X RAY (SES)	Mean± SD	6±0.88	6.2±0.8		0.92	0.36
HAQ	Median(range)	5.8± 1.6	5.9± 1.4		0.002	0.97
RASS	Mean± SD	54.7±9.4	46.6±12.2		15.9	**0.0001**

χ 2: chi square test of significant, p>0.05: insignificant, p<0.05: DAS: disease activity score, SES: short erosion scale, HAQ: health assessment questionnaire, RASS: rheumatoid arthritis severity scale, n: number

Table 3: Frequency distribution of HLA-G rs66554220 (14 bp ins/del) and HLA-G rs1063320 (+3142G>C) polymorphisms in rheumatoid arthritis patients regarding ACPA.

Gene		ACPA positive n.48	ACPA negative n.27	χ^2	p	Odds	95%CI	
							Lower	Upper
HLA-G rs66554220 (14 bp ins/del) (gene)	Gene frequency n (%)			1.5	0.48			
	Ins/ins	10 (20.8)	4 (14.8)	F	0.95	0.91	0.18	4.6
	Ins/del	27 (56.3)	19 (70.4)	F	0.31	0.52	0.14	1.9
	Del/del	11 (22.9)	14.8 (27.5)			Reference		
	Allele frequency n (%)							
	Ins	47 (49)	27 (50)	F	0.99	0.91	0.18	4.6
	Del	49 (51)	27 (50)				Reference	
HLA-G rs1063320 (+3142G>C) (gene)	Gene frequency n (%)			**8.1**	**0.018***			
	GG	37 (77.1)	13 (48.1)	**F**	**0.028***	**11.4**	**1.2**	**111.4**
	GC	10 (20.8)	10 (37)	F	0.34	4	0.38	42.4
	CC	1 (2.1)	4 (14.8)			Reference		
	Allele frequency n (%)							
	G	84 (87.5)	36 (66.7)	F	**0.002***	**3.5**	**1.5**	**8**
	C	12 (12.5)	18 (33.3)				Reference	

χ 2: Chi square test of significant, F: Fisher Exact test, p>0.05 non-significant, *p<0.05: significant, CI: Confidence interval, n: number

Table 4: Frequency distribution of HLA-G rs66554220 (14 bp ins/del) and HLA-G rs1063320 (+3142G>C) polymorphisms in rheumatoid arthritis patients regarding seropositivity for both RF& ACPA.

Gene		RA patients with positivity of both RF& ACPA n.43	Rest of RA patients n.32	χ 2	p	Odds	95%CI	
							lower	upper
HLA-G RS66554220 (14 bp ins/del)	Gene frequency n (%)			2.1	0.36			
	Ins/ins	8 (18.6)	6 (18.8)	F	0.45	0.48	0.1	2.3
	Ins/del	24 (55.8)	22 (68.8)	F	0.25	0.4	0.11	1.4
	Del/del	11 (25.6)	4 (12.4)			Reference		
	Allele frequency n (%)					0.77	0.401	1.47
	Ins	40 (46.5)	34 (53.1)	0.32				
	Del	46 (53.5)	30 (46.9)				Reference	
HLA-G RS1063320 (+3142G>C)	Gene frequency n (%)			**7.6**	**0.02***			
	GG	34 (79.1)	16 (50)	F	**0.053***	**8.5**	**0.88**	**82.5**
	GC	8 (18.6)	12 (37.5)	F	0.62	2.7	0.25	28.5
	CC	1 (2.3)	4 (12.5)			Reference		
	Allele frequency n (%)			**8.8**	**0.03***			
	G	76 (88.4)	44 (68.8)			3.4	1.5	8.04
	C	10 (11.6)	20 (31.2)				Reference	

χ 2: Chi square test of significant, F: Fisher Exact test, p>0.05 non-significant, * p<0.05: significant, CI: Confidence interval, n: number

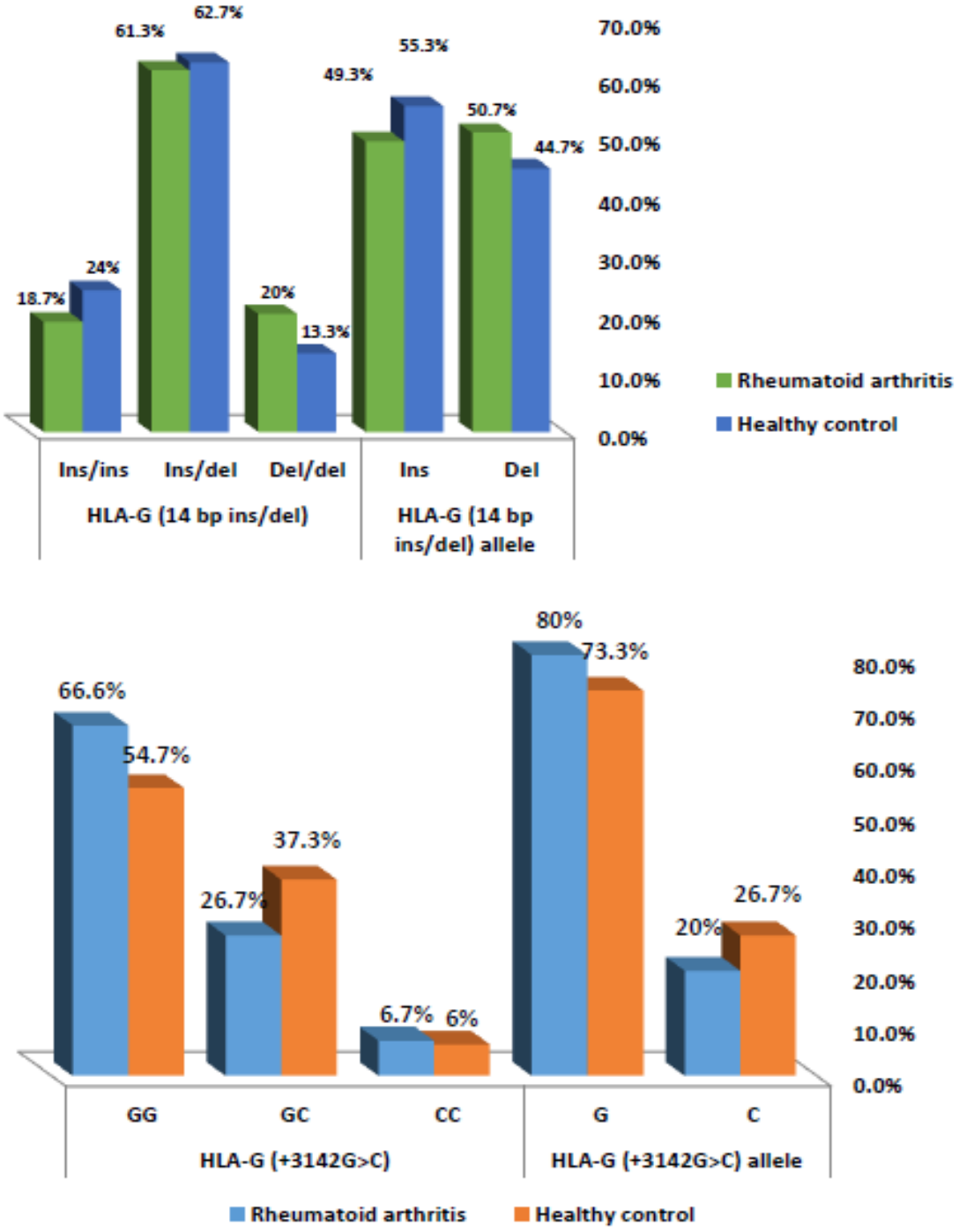

Figure 1: Percent of HLA-G rs66554220 (14 bp ins/del) and HLA-G rs1063320 (+3142G>C) gene and alleles in rheumatoid arthritis patients and healthy controls.

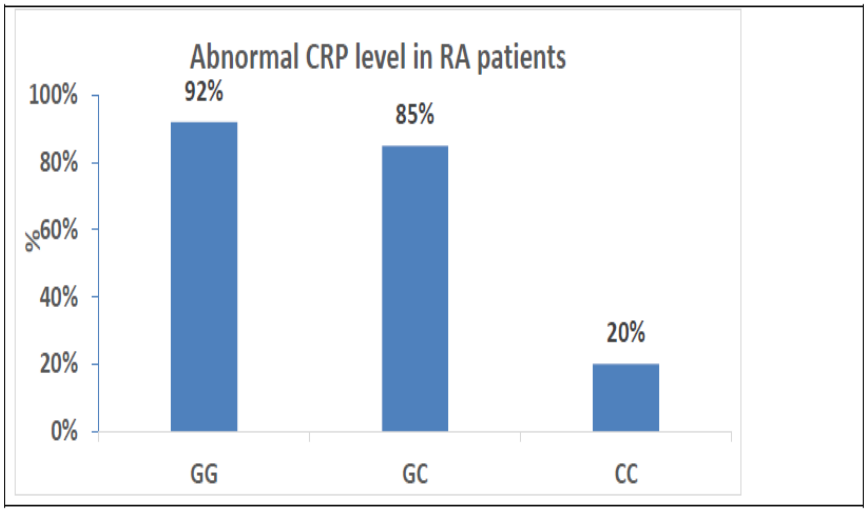

Figure 2: Abnormal CRP level according to HLA-G rs1063320 (+3142G>C) gene in rheumatoid arthritis patients.

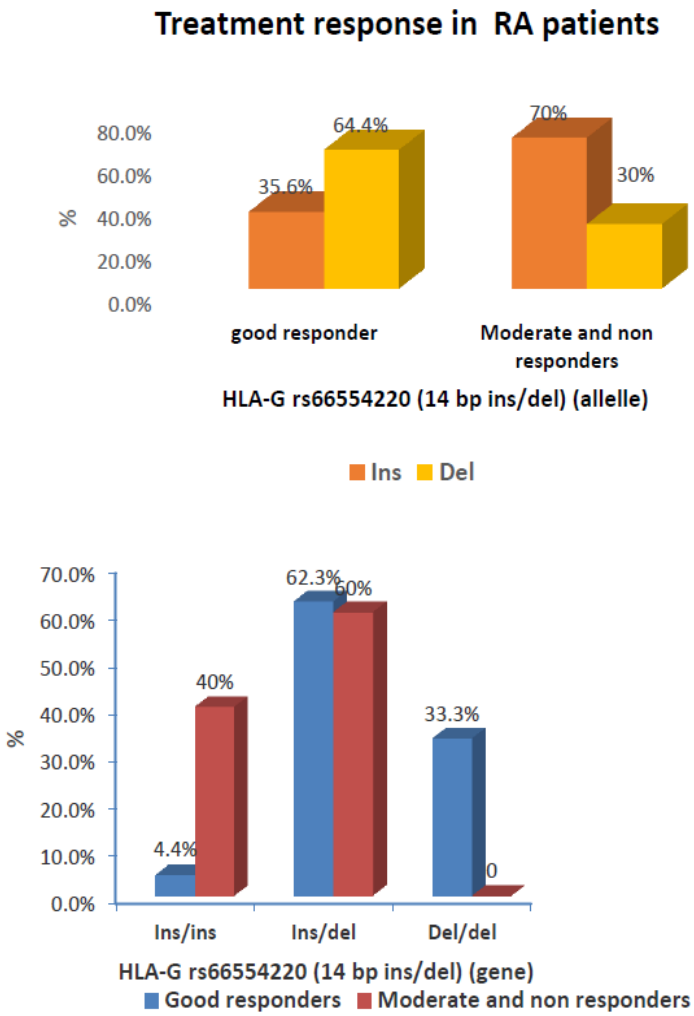

Figure 3: Percent of HLA-G rs66554220 (14 bp ins/del) and HLA-G rs1063320 (+3142G>C) allelle in rheumatoid arthritis patients regarding their treatment response.

DISCUSSION

Immune cells may adhere to the HLA-G molecule, a nonclassical HLA class I molecule, and become inactive [8]. It interacts in several immunoregulatory processes and may have an impact on the pathophysiology of RA. Plasma levels and the pace of HLA-G gene expression are affected by 3'-UTR variations [20, 21].

In the present study, we investigated RA susceptibility, activity, severity, and treatment response were affected by the 14 bp ins/del and +3142G>C polymorphism in a sample of Egyptian patients with RA.

Regarding RA susceptibility, we didn't find any statistically significant differences in either genotype, allele, haplotype and combined analyses of HLA-G 14 pb ins/del and HLA-G +3142G>C variants between patients and controls. Our results are in agreement with Veit et al [22]. They investigated at the genotypic and allelic frequencies of the HLA-G 14 bp ins/del polymorphism and found no differences between controls and RA patients. They did find a connection between juvenile idiopathic arthritis and the 14 bp ins/del polymorphism in the Brazilian population.

In another study, Veit et al [13] reported that the 14 bp ins/del polymorphism and the risk of RA were not shown to be correlated, while the +3142GG genotype significantly increased the risk of RA.

The results of the present study are in line with a meta-analysis conducted by Lee et al [23] that found no significant relationship between RA risk and HLA-G 14 bp ins/del and +3142G/C polymorphism.

Also, similar negative findings have been reported by Catamo et al [24] in Brazilian and Mariaselvam et al [25] in Indian populations.

Our findings are in agreement with Hashemi et al [17] study on Iranian subjects and Gautam et al [10] study on Indian subjects. They discovered no statistically significant differences in the genotype or allele distribution of the HLA-G 14 bp ins/del mutation between patients and controls. However, they also found a connection between RA and the 3142G/C polymorphism.

Hashemi et al [17] showed that RA had considerably greater levels of the GG genotype and G allele. Gautam et al [10] showed that RA patients had a considerably greater rate of the recessive inheritance model (CC genotype compared to GG+ GC) than did controls.

Also, the results of a meta-analysis done by Chowdhury and Lama [26] indicated that there was no significant correlation between the 14 bp ins/del polymorphism and RA susceptibility. The HLA G +3142G>C mutation was found to be significantly associated with RA susceptibility, according to the recessive model.

As regards the effect of HLA G genes, other than included in the present study, Kim et al [27] observed no correlation between the risk of RA in the Korean population and the HLA-G gene promoter polymorphisms rs1736936 (-1202T/C) and rs2735022 (-586C/T). The same unfavourable outcomes were also found by Mariaselvam et al [25] for the rs9380142 (+3187G>A) gene.

Concerning the RA clinical phenotype, the G allele of +3142G/C variant was significantly associated with the presence of subcutaneous nodules and longer morning stiffness.

Using DAS-28, we failed to detect a correlation between HLA-G variations and disease activity in RA. The two HLA-G variations and DAS-28 were also not associated, according to Hashemi et al [17] study.

Patients with the HLA-G+3142G>C GG, GC genotypes, and G allele were considerably more likely to have abnormal CRP levels that were greater than usual.

It has been demonstrated that the GG genotype and the G allele of the HLA-G+3142G>C polymorphism are risk factors for the generation of autoantibodies (ACPA and ACPA+ RF). Both Veit et al [13] and Mariaselvam et al [25] have noted a correlation between the HLA-G+3142G>C status and autoantibody status (RF positive only).

We found that the HLA-G +3142G>C polymorphism is connected to the severity of RA. It was established that the G allele and the GG genotype were linked to higher RASS

scores. This supports the notion that the G would be the 'low producing allele' as it is linked to a higher microRNA affinity and, consequently, to a reduced HLA-G expression. This is in addition to the association of the G allele with the clinical phenotype and high CRP. It could perhaps contribute to the etiology of RA.

Although our results did not demonstrate a link between the HLA-G +3142G>C variant and therapeutic response, we discovered a significant link between the 14 bp ins/del polymorphism and therapeutic response. Patients with the del/del genotype and the del allele had a statistically significant higher percentage of favorable response to treatment (OR =2.4, 95% CI = 1.2-4.6, $p < 0.0001$* for del allele against ins allele). This supports the theory that the HLA-G mRNA isoforms' stability and splicing patterns are influenced by the 14bp ins/del polymorphism, and that the homozygous deletion of 14 bp confers a more stable mRNA, which is associated with higher sHLA-G and a better prognosis because it inhibits the immune response.

Our findings are in agreement with Baricordi et al [28] and Rizzo et al [14]. They observed a correlation between the frequency of the 14 bp del/del genotype and the del allele and disease remission.

Rizzo et al [29], Stamp et al [30], Kooloos et al [31] and the meta-analysis carried out by Lee et al [23] did not show a connection between the HLA-G 14 bp ins/del polymorphism and treatment response, in contrast to our findings. The polymorphism of 14 bp ins/del or HLA-G +3142G>C was also not associated with treatment response, according to Hashemi et al [17].

In contrast to our results, Rizzo et al [29], Stamp et al [30], Kooloos et al [31] and the meta-analysis performed by Lee et al [23] did not demonstrate a link between the HLA-G 14 bp ins/del polymorphism and treatment response. Also, Hashemi et al [17] found no association between the polymorphism of 14 bp ins/del or HLA-G +3142G>C and treatment response.

The present study has some limitations. First, there are the small sample sizes used in subgroup analysis. Second, because we focused on Egyptian patients for our study, only these ethnic groups can use our findings. Third, we lack information on blood HLA-G levels and HLA-DRB.

CONCLUSION

The HLA-G +3142 and the 14 bp polymorphism don't appear to be a risk factor for development of RA in this studied group of RA patients but may act as a genetic modifier of disease phenotype in terms of autoantibody production, severity, clinical phenotype and prediction of good response to treatment. Further studies with larger sample size and different ethnicities are required to verify our findings.

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