ORIGINAL ARTICLE

Role of HLA-G 14 bp polymorphism and soluble HLA-G level in recurrent spontaneous abortion

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ABSTRACT

Background: *The immune response of the mother against her embryo is supposed to be responsible for about 50 % of recurrent spontaneous abortion (RSA) case.* ***Objectives:*** *To assess the prevalence of HLA-G 14bp insertion/deletion (ins/del) polymorphism in females with RSA and normal pregnant females and compare the plasma levels of soluble HLA-G (sHLA-G) in the studying groups. Also, we intended to explore the association between HLA-G 14 bp polymorphism and sHLA-G plasma levels.* ***Methodology:*** *This case-control study involved 50 females with RSA and 50 control pregnant females. The genotype for HLA-G 14 bp (ins/del) polymorphism was performed by PCR and their sHLA-G plasma levels were measured.* ***Results:*** *The HLA-G del/del genotype and ins/ins genotype frequencies were significantly different in RSA group as compared to controls (P=0.002). Additionally, the incidence of the 14-bp ins allele was significantly raised in RSA group than in control (67 vs. 41%, respectively; P=0.0004). Consequently, the higher frequency of 14-bp ins allele in RSA group as compared to controls indicate that this allele is associated with an increased risk of RSA (OR 2.9, 95% CI: 1.6-5.2, P=0.0003).* ***Conclusion:*** *The plasma level of sHLA-G is a promising marker in the management of RSA and could be an early indicator of the fate of In Vitro Fertilization (IVF). The polymorphism in the HLA-G gene, specifically, in the 3′UTR region of exon 8 affects the plasma level of sHLA-G and subsequent pregnancy outcome.*

INTRODUCTION

Recurrent spontaneous abortion (RSA) was described as ≥ 2 repeated miscarriages prior to 20 weeks from the last menstrual period. About 1 in 300 pregnancies presented with RSA[1,2]. The complexed immune response of the mother against her embryo is supposed to be responsible for about 50 % of RSA cases[3]. The immune cells of the mother become into intimate contact with a foreign fetal trophoblast cells throughout pregnancy and the logical result is the rejection which, in fact, does not occur[4]. During conception, the maternal tolerance to the semi-allogenic embryo is linked with expression of HLA-G in fetal-derived cytotrophoblasts at the fetal-maternal interface[5].

The expression of HLA-G exists in seven isoforms: mRNA alternative splicing and differential association with β2microglobulin includes three soluble isoforms (HLA-G5 to HLAG7) and four membrane-bound isoforms (HLA-G1 to HLAG4), however HLA-G1 presents in soluble isoform which can be measured in body fluids by splitting of its membrane-bound isoform from the cell surface by metalloproteinases[6]. The initial HLA G gene polymorphism that includes 14 bp (ins/del) (5′ ATTTGTTCATGCCT 3′) and located in the 3′UTR region of exon 8 of the gene was first described by Harrison and his colleagues[7]. But later numerous regulatory elements, including (poly-A signal and AU-rich motifs) which control mRNA stability and alternative splicing are discovered in this 3′UTR region[8].

Accordingly, HLA-G gene polymorphism that originates from regulated HLA-G gene expression and post-transcriptional modifications, results in variations in the HLA-G expression profile which could be linked with fetal and placental growth[5]. Additionally, researchers reported that the 14-bp insertion allele was accompanied by decreased levels of both sHLA-G isoforms and HLA-G mRNA[9]. Furthermore, it was reported that plasma levels of sHLA-G were intensely decreased with the genotype 14 bp ins/ins than with 14 bp del/del and 14 bp ins/del genotypes[10]. So, the aim of the current study was to evaluate the prevalence of HLA-G 14 bp ins/del polymorphism in the RSA and normal pregnant females with further comparison between their plasma levels of sHLA-G in the studying groups. Moreover, the association between HLA-G 14 bp polymorphism and sHLA-G plasma levels was estimated.

METHODOLOGY

Study design and Subjects

This case-control study was performed at The Microbiology and Immunology Department, Faculty of Medicine, Zagazig University. The study included 2 groups: fifty females presented with recent history of RSA (one week after abortion) recruited from the Outpatient Clinics of the Obstetrics and Gynecology Department, Zagazig University Hospitals, females with abnormal hormonal profile, evidence of autoimmune disorders and any evidence of TORCH infection (toxoplasmosis, rubella, cytomegalovirus and *Herpes simplex* virus) were excluded. Fifty healthy pregnant females, in the first trimester (between 8 and 10th week) were recruited from the antenatal clinic and were included in this study as controls with no history of any obstetric problems.

Ethical Approvals

The Institutional Review Board (IRB) and the ethical committee of Zagazig University Hospitals approved this study. All subjects gave written informed consent before enrollment in this work. Approval number (IRB#4725/26-6-2017).

Genotyping of HLA-G 14 bp polymorphism at exon 8 (3′ UTR)

Genomic DNA was obtained from EDTA-anticoagulated venous blood (2 ml) using a genomic DNA extraction kit **(GeneJET, Thermo Scientific, USA)**, following the manufacturer's guidance. Genotyping of HLA-G 14-bp polymorphism (rs371194629 Chromosome 6 Position(bp) 29,830,804–29,830,805, Accession number: NC_000006.12,Referencesequence:NM_002127.5:c.*65_*66insATTTGTTCATGCCT Genomic position is shown relative to GRCh38.p7; SNP ID is according to dbSNP (rs,http://www.ncbi.nlm.nih.gov/SNP); was done as described by Alegre and his colleagues[11]. Briefly, 100 ng of genomic DNA was amplified in a 25 µL reaction, with an ultimate concentration of the reagents **(iNtRON Biotechnology, Korea)**: 1x Reaction Buffer, 2.5 mM of each dNTP,1.5 mM MgCl2, 2.5 U Taq Polymerase, and 10 pmol of each primer (5'-GTGATGGGCTGTTTAAAGTGTCACC-3') and RHG4 (5'-GGAAGGAATGCAGTTCAGCATGA-3') using a PCR cycler (Biometra). PCR protocol was"95°C for 180 seconds, then 35 cycles of denaturation at 95°C for 60 seconds, annealing at 64°C for 60 seconds and elongation at 72°C for 60 seconds and the last elongation at 72°C for 600 seconds[12]. The investigation of amplified PCR products was done in 3% agarose gel including ethidium bromide (0.5 µg/ml) **(Sigma, USA)** for 40 minutes and then visualized on an UV light using a gel documentation system. The interpretation of the HLA-G 14-bp polymorphism was done by two different observers. Depending on the deletion of the 14bp of exon 8, the amplified PCR products were either of 224 or 210bp, or both 224 and 210 bp (Fig. 1).

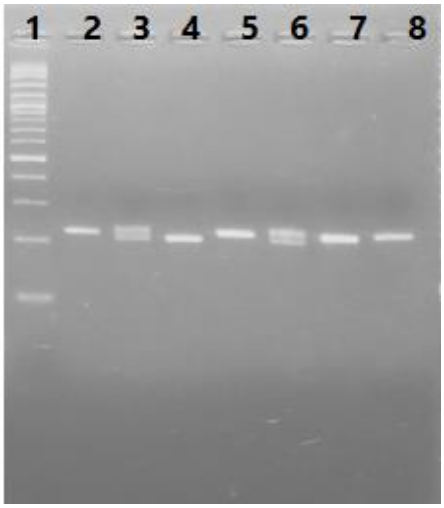

Fig. 1: HLA-G 14 bp polymorphism on agarose gel. Lane 1: 100-bp DNA ladder, Lane 4: HLA-G del/del genotype, Lane 3,6 : HLA-G ins/del genotype, Lane 2,5,7,8: HLA-G ins/ins genotype.

Analysis of sHLA-G plasma levels

Three milliliters of EDTA-anticoagulated venous blood were collected from RSA and control. Plasma was removed following centrifugation (5 minutes at 3000 rpm) of freshly-drawn blood and immediately frozen at −20 C. Levels of sHLA-G antigens were analyzed by sandwich enzyme-linked immunosorbent assay: (ELISA) with sHLA-G ELISA assay kit **(MyBioSource, California, USA)** as stated by the manufacturer's instructions. All tests were done in duplicate; and mean absorbance was estimated for each plasma sample at 450 nm wavelength. The level of sHLA-G is calculated using calibration curves. The extent of sHLA-G ELISA assay kit sensitivity was 0.06U/ml.

Statistical analysis

The sample was calculated to be 100 cases using open-Epi at CI 95% and power of the study is 80%. SPSS (statistical package of social science) version 20 (Chicago, IL, USA)" was used for data analysis. The quantitative results were expressed using mean, median and SD while the qualitative results were expressed in the form of number and percentage. Statistical significance of difference in allele and genotype frequencies between was calculated by Fisher's exact test. The odds ratio (OR) and 95 % confidence interval (CI) were also computed. Hardy-Weinberg equilibrium was estimated for this HLA-G 14 bp polymorphism using the x^2 test[13]. P values less than 0.05 were considered significant.

RESULTS

Table (1) summarizes baseline characteristics of control and RSA groups. Showing that the mean maternal age for RSA group was 31.2 ± 3.65 years, range 25-39 years and the control group mean age was 32.65 ± 4.56 years, range 23-39 years, with no statistically significant difference (P> 0.05) between both groups.

Table 1:Baseline characteristics of control & RSA groups

	Control	RSA group	P value
Patients number	50	50	
Age (mean±SD) years	31.64 ±6.55	32.65 ± 4.56	0.407
sHLA-G Levels (U/mL)			
Median	5.4	1.0	
Range	0.4-10	0.3-3.9	0.004*

SD, standard deviation, * Significant *P* value

HLA-G 14 bp polymorphism

The occurrences of HLA-G 14 bp alleles and genotypes in both control and RSA groups are shown in table (2). The HLA-G del/del genotype and ins/ins genotype frequencies were significantly different in RSA group as compared to controls (P=0.002). Particularly, the ins/ins genotype was more frequent in RSA group (48%) as compared to controls (18%). Additionally, the incidence of the 14-bp ins allele was significantly raised in RSA group than in control (67 vs. 41%, respectively; P=0.0004). Consequently, the higher frequency of 14-bp ins allele in RSA group as compared to controls indicate that this allele is associated with an increased risk of RSA (OR 2.9, 95% CI: 1.6-5.2, P=0.0003) as illustrated in table (2).

Table 2: HLA-G 14 bp allele and genotypes frequencies in control and RSA groups.

HLA-G 14bp	Control group (N=50)		RSA group (N=50)		RSA to control group		P value
Genotypes†	No	%	No	%	OR	C.I (95%)	
del/del	18	36.0	7	14.0	0.289	0.108-0.776	0.011*
ins/ins	9	18.0	24	48.0	4.21	1.69-10.44	0.002*
Heterozygous	23	46.0	19	38.0	1.390	0.629-3.084	0.418
HLA-G 14bp allele‡							
14-bp insertion	41	41	67	67	2.92	1.64-5.2	0.0003*
14- bp deletion	59	59	33	33	034	0.193-0.609	0.0003*

OR; odds ratio, C.I: confidence interval, *P < 0.05 is significant (S). Genotype and allele distributions were compared through Fisher's exact test as following: Control versus RSA (p =0.002†; p =0.0004‡)

Analysis of sHLA-G plasma levels

The results show that the plasma levels of sHLA-G in the RSA group were significantly lower than control group (median concentration= 1.0 U/ml and 5.4 U/ml, respectively, P = 0.004) as illustrated in table (1) with no association between age and the sHLA-G levels (P= 0.55).

Analysis of sHLA-G plasma levels association with HLA-G 14 bp polymorphism

The sHLA-G level was statistically lower in ins/ins genotyping than heterozygous and del/del in both RSA patients and in healthy control group. Among RSA patients, HLA-G levels were 2.4 (U/mL) ,1.5 (U/mL) and 0.4 (U/mL) respectively in del/del, heterozygous and ins/ins genotypes (p<0.0001). While HLA-G levels among control group were 6.6 (U/mL) 3.8 (U/mL) and 0.7 (U/mL) respectively in del/del, heterogenous and ins/ins genotypes (p<0.0001). Table (3) shows a statistically significant difference in sHLA-G level between control and RSA groups according to their genotypes.

Table 3: Levels of sHLA-G (U/mL) among Control and RSA groups according to genotypes.

HLA-G level (U/mL) Median (Range)	Control group	RSA group	P value
del/del	6.6 (2-10)	2.4 (1.4-3.6)	0.000**
ins/ins	0.7 (0.4-3.8)	0.4 (0.3-1.4)	0.007*
Heterozygous	3.8 (0.8-10)	1.5 (0.4-3.9)	0.002*

** Highly significant P value, * Significant P value.

DISCUSSION

During pregnancy, the acceptance of the semi-allogeneic fetal tissue by the maternal immune system is a fundamental issue[14]. The expression of HLA-G and its soluble form is one of the mechanisms that are crucial for the continuation of pregnancy[5]. The effect of sHLA-G on maternal and fetal immune response works by suppressing the maternal T cell and activation of apoptotic pathways of activated CD8 cells[15]. Recurrent spontaneous abortion (RSA) was described as ≥ 2 repeated miscarriages prior to 20 weeks from the last menstrual period[1,2]. RSA is one of the most challenging complications commonly seen in early pregnancy[16].

Correlations between HLA-G polymorphisms and unfavorable pregnancy outcomes have been postulated. The most thoroughly investigated is the 14-bp ins/del polymorphism which affects HLA-G expression, mRNA stability, and alternative splicing[1]. Accordingly, this polymorphic site is an important issue to evaluate adverse pregnancy conditions, especially in RSA.

Regarding HLA-G 14 bp polymorphism, the current study did not find any significant differences in the distribution of the 14-bp in/del genotype between RSA subjects and controls. However, the present article observed a significant increase in the 14-bp ins allele in the RSA group in comparison to control group (P= 0.0004). The occurrence of 14-bp ins/ins genotype was significantly raised in RSA as compared with control women (P= 0.002). These findings are quite similar to other researchers who reported that 14-bp insertion genotype are more frequent in RSA women than normal non-pregnant women[7,17].

Although Al Omar and his collaegues[8] stated similar results in Saudi Arabia, others [18,17] reported more frequency of heterozygous females in RSA group in comparison to normal females. Additionally, other authors observed a greater number of heterozygotes in the controls in comparison to the RSA groups[12]. Unfortunately, this issue may be conflicting but mostly due to significant variations in the geographical, ethnic conditions and linkage disequilibrium with other HLA variants.

Levels of sHLA-G in RSA group were significantly lower than control group (median concentration =1.0 U/ml and 5.4 U/ml, P=0.004) respectively, these findings are similar to other studies, which reported that females with RSA showed lower serum levels of sHLA-G, particularly sHLA-G1 isoform[6,19,20]. Besides, comparable studies indicated that adverse pregnancy outcomes in IVF gestations are associated with low serum levels of maternal sHLA-G[21,22]. Earlier studies also revealed that low sHLA-G in plasma in early pregnancy seemed to be associated with unfavorable outcomes such as RSA and preeclampsia, where immunological factors are thought to play a crucial role[19,23,24].

Although there was a significant difference in sHLA-G levels between RSA and control group, the measured sHLA-G levels were lower than other studies[19]. This could be explained by the use of different methodologies to estimate sHLA-G level. The measuring unit, in our study, was expressed in U/ml[20]. Also, time of collection of samples was totally different.

On the contrary, others; Mubarak and his colleagues[25] reported that serum sHLA-G levels were higher among pregnant females suffering from RSA in comparison to normal pregnant women. In both groups, sHLA-G levels were also elevated in the second trimester in parallel to first trimester. These variations may base on ethnic and genetic variation as the study was conducted on African subjects.

The low levels of sHLA-G in RSA group could be attributed to changes in cytokine profile in abortion as decreased IL-10, which is well known as a stimulator of HLA-G production[26]. Interleukin-10 has been shown to induce HLA-G expression[27]. Another factor is the fetal genotype which should be considered to understand the actual role of HLA-G in the outcome of a pregnancy. Dahl and his colleagues found that increasing numbers of fetal 14 ins alleles are related to significantly increased levels of sHLA-G in maternal blood plasma samples at term in heterozygous mothers and concluded that combined fetomaternal HLA-G genotypes are related to sHLA-G levels in maternal blood plasma[28]. Interestingly, Pfeiffer and his colleagues found that from the 8th GW sHLA-G levels in women with an intact twin pregnancy increased significantly versus singleton pregnancy points to the possible presence of fetally derived sHLA-G molecules in maternal blood[21].

Concerning the distribution of sHLA-G levels among the studied RSA and control groups according to HLA-G 14 bp polymorphism, The sHLA-G level was statistically lower in ins/ins genotyping than heterogenous and del/del in both RSA patients and in healthy control group, this comes in line with previous studies[11,29,30]. Rebmann and his colleagues postulated that the (+14b) alleles are 'low secretor' alleles that are associated with low plasma level of sHLA-G while 'high secretor' HLA-G allele (-14b), was shown to be associated with elevated plasma level of soluble HLA-G. Several studies clarified this finding by that the insertion of 14 bases may yield the cutting of 92 bases in a fraction of the primary transcript, eliminating at least two polymorphic sites in the HLA-G 3' UTR and giving rise to shorter mRNAs with increased stability[31]. The loss of 92 bases of the primary transcript eliminates a region that may be an important target for microRNAs, which could bind to and inhibit translation or reduce the stability of mRNA[10,32]. In an interesting sequencing study, Martelli-Palomino and his colleagues observed that that other polymorphic sites located at the HLA-G 3' UTR were also associated with the levels of sHLA-G, including +3142 C/G , +3187 A/G, +3010

C/G, +3027 A/C and +3035 C/T genotypes[10]. This is a vital issue for future research.

CONCLUSION

The plasma level of sHLA-G is a promising step in the diagnosis of RSA and could be an early indicator for the fate of IVF. The polymorphism in the HLA-G gene, specifically in the 3′UTR region of exon 8 affects the plasma level of sHLA-G and subsequent pregnancy outcome.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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