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Original Article

Evaluation of HLA-G 14-bp ins/del and +3142G>C polymorphisms with susceptibility to recurrent spontaneous abortion

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ABSTRACT

Objective: HLA-G is critically important for successful implantation during pregnancy. Increasing evidence supposed that HLA-G plays a key role in tolerance of the semi-allogeneic graft in pregnancy by inhibiting the cytotoxic functions of T and NK cells. The present study aimed to evaluate the impact of HLA-G rs1063320 (+3142G>C) and 14-bp insertion (ins)/deletion (del) polymorphisms on recurrent spontaneous abortion (RSA).

Materials and Methods: Genomic DNA from 93 RSA patients and 93 normal fertile women was isolated using the salting out method. Genotyping of HLA-G +3142G>C and 14-bp ins/del variants was done by polymerase chain reaction restriction fragment length polymorphism (PCR-RFP) and PCR method, respectively.

Results: The *HLA-G* +3142G>C polymorphism increased the risk of RSA in codominant (OR = 2.39, 95% CI = 1.27–4.49, p = 0.010, GC vs GG; OR = 3.28, 95%CI = 1.16–9.72, p = 0.040, CC vs GG) and dominant (OR = 2.52, 95%CI = 1.37–4.64, p = 0.004, GC + CC vs GG) tested inheritance models. *HLA-G* rs1063320 C allele was associated with increased risk of RSA (OR = 1.84, 95%CI = 1.20–2.83, p = 0.007). The del/del genotype as well as del allele of 14-bp ins/del variant increased that risk of RSA (OR = 3.02, 95%CI = 1.23–7.41, p = 0.025 and OR = 1.65, 95%CI = 1.09–2.50, p = 0.022, respectively).

Conclusion: In summary, our results showed that *HLA-G* gene polymorphisms significantly increased the risk of RSA in a sample of the Iranian population.

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Introduction

Miscarriage is one of the most common complications of pregnancy, which accounts for about 10–15% of clinically recognized pregnancies [1,2]. It is estimated that 1–3% of women may have more than three consecutive unexplained pregnancy losses before the 20th week, which is termed recurrent spontaneous abortion [3]. However, many researchers have now revised the definition to two or more consecutive RSA [4].

The pathomechanism of RSA is still not entirely understood. The disorders of immune homeostasis between the fetus and the maternal immune system are considered as one of the potential etiological factors for RSA [5]. The maternal immune system is in close contact with cells and tissue from the semi-allogenic fetus during pregnancy. Consequently, there must be precise mechanisms to modulate the maternal immune system to avoid rejection of semi-allogenic fetus. It has been proposed that the expression of non-classical HLA proteins at the feto-maternal interface possibly play a key role in the maintenance of pregnancy [6,7]. HLA-G is unique among class I genes because it undergoes alternative splicing to produce four encoding membrane-bound proteins (HLA-G1–G4) and three soluble proteins (HLA-G5–G7). At least four of these splice forms (two membrane-bound and two soluble) are expressed as protein in fetal cells at the feto-maternal interface

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[8–10] which may contribute to maternal tolerance of the semi-allogenic fetus [11–14]. HLA-G gene is located on chromosome 6 (6p21.31). It has been reported that HLA-G expression is influenced by 14-bp insertion (ins)/deletion (del) and a +3142G/C (rs1063320) polymorphisms in the 3′ untranslated region (3′UTR) of HLA-G gene and may have possible implications of clinical significance [15]. The studies focusing on the HLA-G polymorphisms with RSA have been largely inconclusive [16–19]. Due to genetic risks may vary among diverse populations, repeating previously reports of association of HLA-G variants and RSA in other population is required to find out the genetic risk. Therefore, the present study aimed to find out the impact of HLA-G rs1063320 (+3142G>C) and 14-bp ins/del polymorphisms on SRA in a sample of Iranian population.

Material and methods

Patients

A total of 93 patients with two or more recurrent spontaneous miscarriages (as a case group) and 93 healthy women without any history of miscarriage and had at least one normal birth (as a control group) were recruited to the study. Recurrent miscarriage was defined as two or more consecutive pregnancy losses before 20 weeks of gestation. In addition, all of the patients were without anatomical, microbial, viral, hormonal and genetic disease. The project was approved by local ethics committee of Zahedan University of Medical Sciences and informed consent was obtained from all participants. Genomic DNA was extracted from peripheral blood samples using salting out method as described previously [20].

Genotyping of HLA-G rs1063320 (+3142G>C) variant was performed by PCR-RFLP methods. The set of forward and reverse primers were 5′-CATGCTGAACTGCATTCCTTCC-3′ and 5′-CTGGTGGGACAAGGTTCTACTG-3′. Amplification was done with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 30 s at 95 °C, 30s at 65 °C and 30s at 72 °C with a final step at 72 °C for 10 min. Ten μl of PCR products digested with BaeGI restriction enzyme (Fermentas). G allele digested and produced 316-, and 90-bp (digested) while C allele undigested and produced 406-bp.

Genotyping of HLA-G 14-bp ins/del variant was done by polymerase chain reaction [21]. The forward and reverse primers were 5′-TCACCCCTCACTGTGACTGATA-3′ and 5′-GCA-CAAAGAGGAGTCAGGGTT-3, respectively. In each 0.20 ml PCR reaction tube, 1 μl of genomic DNA (~100 ng/ml), 1 μl of each primer (10 μM), 10 μl of 2X Prime Taq Premix (Genet Bio, Korea) and 5 μl ddH2O were added. The PCR cycling conditions were as follows: an initial denaturation step of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, with final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis in 2% agarose gels, and observed under ultraviolet light. Product sizes were 127-bp for del and 141-bp for ins allele.

Statistical analysis

Statistical analysis of the data was done using statistical software package SPSS 20 software. Independent sample t-test for continuous data and $\chi 2$ test for categorical data were used. The associations between genotypes of *HLA-G* gene and RA were assessed by computing the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. p-value less than 0.05 were considered statistically significant. We estimated the Hardy Weinberg equilibrium (HWE) separately for cases and controls.

Results

In this study, we recruited 93 miscarriage patients age 28.88 ± 4.98 years and 93 unrelated healthy women (30.01 ± 4.77, years). There was no significant difference between the groups concerning age (p = 0.815).

In Table 1, the genotype and allele frequencies of *HLA-G* polymorphisms in cases and in controls are shown. The *HLA-G* rs1063320 (+3142G>C) polymorphism increased the risk of SRA in codominant (OR = 2.39, 95%CI = 1.27–4.49, p = 0.010, GC vs GG; OR = 3.28, 95%CI = 1.16–9.72, p = 0.040, CC vs GG) and dominant (OR = 2.52, 95%CI = 1.37–4.64, p = 0.004, GC + CC vs GG) tested inheritance models. *HLA-G* rs1063320 C allele was associated with increased risk of SRA (OR = 1.84, 95%CI = 1.20–2.83, p = 0.007).

Regarding 14-bp ins/del variant, the findings indicated that del/del genotype increased that risk of SRA compared to ins/ins genotype (OR = 3.02, 95%CI = 1.23–7.41, p = 0.025). The del allele significantly increased the risk of SRA (OR = 1.65, 95% CI = 1.09–2.50, p = 0.022) compared to ins allele.

Haplotype analysis (Table 2) indicated that haplotypes Cdel and Cins increased the risk of SRA in comparison with rs1063320 G/14-bp ins (OR = 2.22, 95%CI = 1.33–3.71, p = 0.0026 and OR = 12.76, 95%CI = 1.47–110.78, p = 0.022, respectively).

The genotype of HLA-G +3142G>C and 14-bp ins/del variants in controls were in HWE ($\chi 2$ = 0.178, P = 0.673 and $\chi 2$ = 0.715, P = 0.397, respectively).

Discussion

Mounting evidence revealed that HLA-G may play a role in the suppression of immune responses and contribute to long-term immune escape or tolerance [22–24]. HLA-G is a non-classical HLA class I molecule can bind to immune cells and inhibit their function [25,26]. The 3'-untranslated region (UTR) has a major role in HLA-G regulation [27,28].

Table 1
Association of HLA-G gene polymorphisms and the risk of recurrent spontaneous abortion.

HLA-G polymorphisms	Case n (%)	Control n (%)	OR (95%CI)	p
rs1063320				
Codominant				
GG	26 (28.0)	46 (49.5)	1.00	–
GC	54 (58.1)	40 (43.0)	2.39 (1.27–4.49)	0.010
CC	13 (14.0)	7 (7.5)	3.28 (1.16–9.27)	0.040
Dominant				
GG	26 (28.0)	46 (49.5)	1.00	
GC + CC	67 (72.0)	47 (50.5)	2.52 (1.37–4.64)	0.004
Recessive				
GG + GC	80 (86.0)	86 (92.5)	1.00	
CC	13 (14.0)	7 (7.5)	2.00 (0.76–5.26)	0.237
Allele				
G	106 (57.0)	132 (71.0)	1.00	–
C	80 (43.0)	54 (29.0)	1.84 (1.20–2.83)	0.007
14-bp ins/del				
Codominant				
ins/ins	20 (21.5)	33 (35.5)	1.00	–
ins/del	51 (54.8)	48 (51.6)	1.75 (0.89–3.46)	0.146
del/del	22 (23.7)	12 (12.9)	3.02 (1.23–7.41)	0.025
Dominant				
ins/ins	20 (21.5)	33 (35.5)	1.00	
ins/del + del/del	73 (78.5)	60 (64.5)	2.01 (1.05–3.85)	0.051
Recessive				
ins/ins + ins/del	71 (76.3)	81 (87.1)	1.00	
del/del	22 (23.7)	12 (12.9)	2.09 (0.97–4.53)	0.088
Allele				
ins	91 (49.0)	114 (61.3)	1.00	–
del	95 (51.0)	72 (38.7)	1.65 (1.09–2.50)	0.022

Table 2
Haplotype frequencies of HLA-G polymorphisms in recurrent spontaneous abortion and control women.

rs1063320 G>C	14-bp ins/del	Cases (frequency)	Controls (frequency)	OR (95%CI)	P
G	ins	0.4397	0.6160	1.00	–
C	del	0.3913	0.2827	2.22 (1.33–3.71)	0.0026
G	del	0.1248	0.0951	1.93 (0.95–3.90)	0.071
C	ins	0.0441	0.0062	12.76 (1.47–110.78	0.022

In the present study we aimed to find out the impact of HLA-G variants and risk of RSA in a sample of Iranian population. The findings revealed an association between HLA-G +3142G>C as well as 14-bp ins/del polymorphism and risk of RSA. HLA-G is involved in maternal-fetal immune tolerance and is reported to be a natural tolerogenic molecule. The major isoform, HLA-G1 (and its soluble type HLA-G5), binds to the inhibitory immune receptors, leukocyte immunoglobulin (Ig)-like receptor (LILR) B1 and LILRB2. It has been shown that sHLA-G expression is significantly reduced in women with RM, and it is significantly elevated in pregnant women in comparison with non-pregnant women [29]. It has been proposed that polymorphisms exert a significant effect in the HLA-G function and may impact on the expression of sHLA-G [30,31,15]. These data also indicate that HLA-G molecules play a critical role in pregnancy outcome.

Zidi et al. [32] have found that sHLA-G1 and HLA-G5 levels are decreased in Tunisian women with multiple abortion. A meta-analysis performed by Fan et al. [17] did not support an association between the HLA-G 14-bp ins/del polymorphism and the risk of recurrent miscarriage (RM). But this variant was associated with RM risk in patients with three or more miscarriages. Controversy exists on the number of miscarriages needed to define RM. In some studies, RM defined as the loss of three or more consecutive pregnancies while other studies define RM as at least two consecutive pregnancy losses [33,34]. Al Omar et al. [18] investigated the impact of HLA-G 14-bp ins/del polymorphism and the recurrent spontaneous abortions in Saudi Arabian women. They found no significant association between the variant and risk of RSA. Agrawal et al. [16] have found that mutant genotypes of -1179G>A (rs1233335), -725C>G/T (rs915670) and -486A>C (rs114252012) variants of HLA-G significantly increased the risk of RSA in Indian women. Arjmand et al. [19] reported that HLA-G*0105N allele significantly increased the risk of RSA in women from Yazd, Iran. While, their findings did not support an association between HLA-G rs1736936 variant and RSA risk. Afkhami et al. [35] have found an association between HLA-G 14-bp ins/del variant and risk of RM.

Some studies have observed that 14-bp ins/ins genotype was associated with RM [36–39]. While the results of some studies did not support an association between HLA-G 14-bp ins/del polymorphism and risk of RM [40–42]. Recently, Michita et al. [34] reported that HLA-G 14-bp ins/del variant was not associated with the risk RSA and the +3142CC genotype as well as C allele decreased the risk of RSA in a Brazilian population. Nowak et al. [43] have found no significant association between HLA-G 14-bp ins/del variant and risk of RSA in Polish population.

In our study, the cases and controls carefully selected on the basis of their clinical characteristics, age and ethnicity. In accordance with other studies [18,34,40,41,43], the patients with two or more pregnancy loss before 20 weeks of gestation were considered as RSA.

The findings of the current study are compared with other studies (Table 3), there is no clear reason for controversial findings among various studies with respect to the HLA-G polymorphisms. It may be attributed in the different distribution of the polymorphism due to ethnicity of the groups under study, and probable linkage disequilibrium with other HLA variants.

In summary, we found a significant association between HLA-G rs1063320 (+3142G>C) and 14-bp ins/del variants and risk of RSA. Further association studies with large sample size and different ethnicities are needed to confirm our findings.

Table 3
Distribution of genotypes of HLA-G 14-bp ins/del and rs1063320 (+3142G>C) polymorphisms in RSA and controls in different populations.

Study	Total sample size (case/control)	14-bp ins/del						p
		RSA			Control			
		ins/ins	ins/del	del/del	ins/ins	ins/del	del/del	
Tripathi et al. [42]	120/120	23	70	27	32	51	37	0.049
Yan et al. [41]	79/109	35	37	7	33	57	19	0.074
Xue et al. [39]	24/88	1	17	6	6	38	44	0.055
Zhu et al. [37]	326/251	53	111	162	29	98	124	0.203
Afkhami et al. [35]	85/85	3	74	8	14	55	16	0.002
Nowak et al. [43]	287/219	56	121	100	35	110	74	0.285
Michita et al. [34]	133/152	30	62	41	23	71	58	0.202
Al Omar et al. [18]	64/62	18	33	13	14	35	13	0.768
Hviid et al. [44]	61/47	15	27	19	5	30	12	0.082
Agrawal et al. [16]	100/100	30	52	18	21	35	44	<0.001
Aruna et al. [45]	141/151	34	67	40	33	87	30	0.152
Akhtar et al. [46]	25/25	4	15	6	6	10	9	0.367
Jassem et al. [40]	49/48	22	17	10	14	21	13	0.275
Current study	93/93	20	51	22	33	48	12	0.045

HLA-G rs1063320 (+3142G>C)

Study	Case/control	RSA			Control			p
		GG	GC	CC	GG	GC	CC	
Jassem et al. [40]	49/48	26	16	7	23	19	6	0.775
Michita et al. [34]	133/152	46	64	23	39	73	40	0.105
Current study	93/93	26	54	13	46	40	7	0.009

Compliance with ethical standards Funding

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Conflict of interest

None of the authors of this study has any conflict of interest to declare.

Ethical approval

All procedures performed were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments. Institutional review and approval was done by the Zahedan University of Medical Sciences. Informed consent was taken from all individual participants included in the study.

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