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Original Article

HLA-G Polymorphic Variation (G*0104N exon 3) Confers Potential Risk for Recurrent Pregnancy Losses: A Study in a High Incidence Zone (Kashmir, North India)

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Abstract

Background and objectives: *HLA-G* gene harbors certain polymorphic variations that can potentially impact its biological activity, and therefore, may confer a risk for recurrent pregnancy loss (RPL). This study aimed to analyze whether *HLA-G* polymorphic variations (*G*0103, G*0104,* and *G0105N*) are related to the risk of RPL in women from Kashmir, North India.

Methods: A total of 200 women who suffered ≥2 RPLs and 240 healthy controls were recruited from the same geographical region. Additionally, 100 spouses of RPL affected women and 60 products of conception were evaluated. *HLA-G* genotyping was performed using the polymerase chain reaction-restriction fragment length polymorphism method.

Results: The variant genotype 0103:0103 in exon 2 of *HLA-G* was not detected. The genotype 0104/0105 was detected in 100% of RPL patients, spouses, and controls. Exon 2 and variant genotypes G*0103 in exon 2 and G*0105 in exon 3 of *HLA-G* were absent in our population and thus did not contribute to the etiopathogenesis of RPL. In contrast, the exon 3 *HLA-G* variant G*0104N was significantly more frequent in RPL patients and their spouses compared to the control group (p<0.05). The presence of the *HLA-G* variant genotype G*0104N (exon 3) was detected in 13% of RPL patients and 7% of their male partners, indicating a significantly higher frequency than in controls and suggesting a substantial risk for RPL (p<0.05).

Conclusions: This study revealed that the higher frequency of the *HLA-G*0104* allele in both partners strongly predicted a substantial risk for RPL in our population.

Introduction

Recurrent pregnancy losses (RPL) due to recurrent miscarriage (RM) are a significant reproductive problem affecting approximately 2% of women in the general population.[1] RM is a common complication of pregnancy, accounting for approximately 10–15% of pregnancy losses, with nearly 80% occurring within the first trimester of gestation.[2] The latest consensus also defines RPL as the occurrence of two or more successive losses of pregnancy before 28 weeks of gestation.[3] Globally, RPL is recognized as a significant reproductive health concern, with more than 50% of couples experiencing RPL having no apparent known cause.[4] The intricate regulation between the fetus and the maternal body, involving numerous mediators such as cytokines, histocompatibility antigens, hormones, lifestyle factors, genetic factors, and angiogenic factors,[2,5–8] is crucial for viable pregnancy. Genetic polymorphisms that impact maternal and fetal factors are believed to be associated with the risk of RPL.

HLA-G, a non-classical HLA class I antigen, is expressed by invasive cytotrophoblasts and likely plays an essential role in generating a tolerogenic state at the feto-maternal interface.[9] *HLA-G* influences maternal acceptance of the semi-allogenic fetus by

Keywords: *HLA-G*; Major histocompatibility complex; Recurrent pregnancy loss; Full-term pregnancy; Exon 2/3; Product of conception.

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adapting the maternal immune system during pregnancy, making it an important immune-tolerant factor that inhibits immune cell functions such as natural killer cells, T-lymphocytes, and dendritic cells.[10–13] *HLA-G* transcripts are significantly found in placental tissue during the first trimester, predominantly in the extravillous membranes, validating the theory that *HLA-G* plays an important role in fetal protection.[14,15]Additionally, *HLA-G* is thought to be involved in pregnancy complications such as preeclampsia and RPL.[16–19]

HLA-G gene, present on the short arm of chromosome 6, contains various polymorphic sites in both coding and non-coding regions that potentially influence its biological functions, including the modulation of the immune response.[20] Alternative splicing of the primary *HLA-G* transcript yields seven *HLA-G* isoforms, with three soluble isoforms (*HLA-G* 5, -G 6, and -G 7) present in large quantities in the maternal circulation during gestation.[15] Studies have shown that plasma levels of soluble *HLA-G* (s *HLA-G*) secreted by a class of immune cells are lower in early miscarriage compared to normal pregnancy, indicating that s *HLA-G* molecules are likely to play a vital role in embryo implantation.[21,22] In pregnancies with *in vitro* fertilization, low levels of s *HLA-G* in maternal circulation concurrent with unfavorable pregnancy outcomes, compared to embryos that secrete sufficient s *HLA-G,* result in successful pregnancy.[18] Several investigations on *HLA-G* gene polymorphisms have revealed relationships between different *HLA-G* alleles and RPL.[23,24] The presence of *HLA-G*0104* and *HLA-G*0105N* alleles in either member of the couple has been strongly associated with an elevated risk of RPL.[25] Furthermore, evidence from a few investigations has established the potential of *HLA-G* as a possible target for the therapeutic function.[26,27]

Several studies have substantiated that elevated serum *HLA-G* levels are related to decreased risk of RPL.[28,29] Our observational study revealed a high prevalence of RM in the Kashmiri population, particularly in the younger age group. Our population strongly favors the custom of consanguineous marriages, which may lead to an increased prevalence of severe genetic disorders, including RPL, over multiple generations. Given the credible role of *HLA-G* in pregnancy outcomes, the present study was conducted to explore the association between *HLA-G* genotype and the patients who suffered RPL compared to normal healthy fertile women with successful full-term pregnancies.

Materials and methods

The study enrolled 200 females who experienced ≥2 pregnancy losses due to RM, along with their corresponding male partners for *HLA-G* (*0103,*0104,*0105N). Additionally, 60 tissue samples from products of conception (POC: abortuses) were examined for *HLA-G* polymorphism. The study was conducted between 2014 and 2019 at the Advanced Centre for Human Genetics, SK Institute of Medical Sciences (SKIMS), J&K (North India), and all patients were referred from the Department of Obstetrics and Gynecology (SKIMS). Utmost care was taken to ensure that the patients strictly fulfilled the fundamental diagnostic criteria of RM, and all groups of RPL patients were enrolled either before or at the 20th week of gestation. A detailed pedigree analysis was performed to identify patients with a multigenerational history of RPL. The patients' blood reports, including hormonal, immunological, biochemical profiles, and radiological investigations, were recorded. Patients were recruited after obtaining their written informed consent, and the study was approved by the Institutional Ethics Committee of SKIMS under protocol No. SKIMS Study ref:81/2014. To exclude other causes of recurrent abortion, patient details were recorded for radiological imaging, cytogenetic reports, related infection tests, and immunological profiles. A total of 240 women matched for age and geographical location with at least two live births and without a previous history of abortion were enrolled as the control group. Approximately 2 ml to 3 ml of venous blood from both groups was collected in Ethylenediamine tetraacetic acid (EDTA) vials and stored at −80°C until further processing for DNA extraction. Using nMaster 2.0 statistical software, the sample size of the study was calculated to be >80%. The clinicopathological characteristics of the patients and controls are shown in Table S1.

Extraction of genomic DNA

DNA extraction from the blood samples of both patients and controls was performed using the phenol-chloroform method and a DNA Extraction Kit (Qiagen).

HLA-G genotyping by PCR-RFLP

Genotyping of *HLA-G** 0103, 0104, and 0105N was carried out by PCR-RFLP. Exons 2 and 3 of *HLA-G* encompassing G*0103, G*0104 and G*0105N alleles were amplified by PCR using the following primers (Biotools, B & M Labs, Madrid, Spain): Exon 2 Forward (F): 5′-TCCATGAGGTATTTCAGCGC-3′, Reverse (R): 5′-CTGGGCCGGAGTTACTCACT-3′; Exon3: F: 5′-CACA CCCTCCAGTGGATGAT-3′R: 5′-GGTACCCGCGCGCTGCAG-CA-3′. For Polymerase chain reaction (PCR), the optimal reagents were used as follows: DNA (100–250 ng/mL), 1×PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$), deoxyribonucleotide triphosphate (Biotools, B & M Labs, Madrid, Spain): 10 mM each of dATP, dCTP, dGTP, dTTP) (Sigma-Aldrich, USA): 10 pM in sterile deionized water), and 1 unit of *Taq DNA polymerase* (Biotools, Madrid, Spain). The PCR thermal conditions for *HLA-G* exon 2 and 3 included 35 cycles: 94°C for 30 sec, annealing at 57°C for exon 2 and 61°C for exon 3 for 30 sec, and 72°C for 30 sec with a final extension at 72°C for 7 minutes. Amplified products (10µL) from exon 2 and exon 3 were digested with the restriction endonuclease *Hinf-I* for exon 2 and exon 3 with *PpuM-I*, and *BseR-I* (Fig. S1). The interpretation of various genotypes is provided in Table S2.

Statistical analysis

All the statistical analyses were performed using IBM Statistics SPSS software (Version-23). Both cases and controls were analyzed by the chi-square test for categorical variables, such as gender and age. To assess whether the polymorphisms were in Hardy-Weinberg equilibrium between patients and controls, we applied a goodness-of-fit chi-square test. The relative risk was estimated in terms of odds ratios (ORs) and 95% confidence intervals (CIs) to observe the association between *HLA-G* genotypes or other related risk factors and RM. Statistical significance was set at $p < 0.05$.

Results

The cases and controls were frequency-matched with respect to age and family history ($p > 0.05$). RM cases with <3 abortions accounted for 46% (92) while 54% (108) accounted for ≥3 miscarriage events. RPL cases with consanguinity and family history were seen in 28.5% and 16% respectively (Table S1).

Genotyping of exon 2 genotype 0103:0103

Among the 200 RM patients and 240 healthy controls with full-term pregnancies, the variant genotype of exon 2 of *HLA-G*,

Table 1. ***HLA-G*** **Exon 2 and Exon 3 genotypes observed in Recurrent Abortion cases and healthy controls**

Parameters	***HLA*** **exon 2 and 3 genotype**	**Cases females N 200 (%)**	**Control 240 (%)**	***p*-value**
Overall genotype	*HLA-G**0103	0	0	Ref
	All but*0103	200 (100)	240 (100)	-
	*HLA-G**0104:0104	13 (7)	2 (0.8)	Ref
	*HLA-G**0104:0105N	187 (93)	238 (99.2)	<0.05
	*HLA-G**0105N: 0105N	0	0	-
	All but G*0105N	200 (100)	240 (100)	Ref
Allele	0103	0	0	-
	0104/0105	400 (100	480 (100)	Ref
	G*0105N	187	0	<0.05
	G*0104	52	14	-
	G*0103/0104	400	480	

0103:0103, was absent in our population. However, the frequency of *HLA-G* genotype 0104/0105 was 100% in both the RPL patients and the control group (Table 1). Additionally, the spouses of RPL patients showed a 100% frequency of the 0104/0105 *HLA-G* genotype. Furthermore, genotyping of 60 POC samples for *HLA-G* exon 2 revealed 100% frequency of the 0104/0105 *HLA-G* genotype, while the variant genotype 0103/0103 was not found in our population (Table 2).

Genotyping of exon 3 Ppum I restriction site (0105:0105 genotype)

For the *Ppum* I restriction site of exon 3 of *HLA-G,* as described in Table 1, the same series of RPL patients and full-term healthy controls underwent polymorphic analysis. Genotype 0105:0105 was not detected in either patients or healthy controls. Conversely, the frequency of *HLA-G* genotype 0104:0103 was 100% in both groups. Moreover, a similar scenario was found in the spouses of the RPL patients (100 samples), which showed the absence of the 0105:0105 genotype in our population, but the frequency of the other variant genotype 0104:0103 was 100% in the same subjects. Similarly, fetal abortuses exhibited the same pattern, wherein all 60 POC samples examined showed a 100% frequency of the 0104:0103 *HLA-G* genotype, while the other variant (0105:0105) was completely absent (Table 3).

*Genotyping of exon 3 BseR*I restriction site (0104:0104 genotype)

As shown in Table 2, for exon 3 restriction site *BseR*I, the frequency of *HLA-G* 0104:0104 was 13% in RPL patients vs. 0.8% in controls, whereas the frequency of the *HLA-G* 0103:0105 variant was 87% in patients compared to 99.2% in controls, with a significant association between the two groups ($p<0.00001$). Similarly, among spouses belonging to corresponding RPL patients, 93% showed the 0103:0105 *HLA-G* variant, with a significant difference compared to controls ($p<0.05$). Interestingly, the control population exhibited a frequency of 99.2% for the 0103:0105 genotype, while the other genotype, 0104:0104 was present in only 0.2% of the patients. In exon 3, all genotypes except G0105N were significantly associated with RPL (100%).

Overall, exon 2 and exon 3 *HLA-G* variant genotypes G*0103 and G*0105, respectively, did not exist in our population and thus have no role in the etiopathogenesis of RPL. In contrast, the frequency of the exon 3 *HLA-G* variant G*0104N was significantly greater in RPL patients than in their spouses but was negligible in the control group ($p<0.05$). The presence of the 13% *HLA-G* variant genotype G*0104N (exon 3) in RPL patients and 7% in their male partners together was significantly greater frequency than that in controls, which can pose a substantial risk for pregnancy losses ($p<0.05$). All parameters related to RPL were stratified, and their associations are depicted in Table 2.

Discussion

HLA-G is a distinctive immunomodulator expressed in various tissues, and its polymorphic variations have been associated with a spectrum of diseases, from autoimmune diseases to cancer.[30–32] *HLA-G* is exclusively expressed at the maternal-fetal interface in fetal tissues and plays a crucial role in the functional activities of the local maternal immune response. This distinctive expression pattern, particularly in *HLA* genes such as *HLA-G,* is believed to be significant for the preservation and maintenance of pregnancy.[33–36] *HLA-G* is considered vital in obstetric complications, primarily including RM and preeclampsia.[13–16] In this study, we analyzed the role of *HLA-G* to observe the association between *HLA-G* genotypes and RPL patients, wherein the *HLA-G* G*0104N (exon 3) variant genotype was found to pose a substantial risk for pregnancy losses.

The current study identified the *HLA-G**0104 allele as a significant predictor of the risk of RPL. The frequency of *HLA-G*0104* was 13% in RPL patients compared to 0.8% in healthy controls. This finding aligns with Aldrich *et al.,*[21] who demonstrated a novel relationship between the *HLA-G*0104* allele and RPL. Located in the α2-domain, *G*01040x* alleles are non-synonymous polymorphic variants of *HLA-G* that have shown an impact on the pregnancy outcome in our study, with an estimated frequency of 13% in females and 7% in their male partners, compared to a negligible presence (0.8%) in healthy controls. However, Matter *et al.*[37] reported a difference in the frequency of *HLA-G*0104* between RPL patients and controls (17.5% and 12.5%, respectively) but did not observe significant differences between the two groups. Conversely, some reports have shown no association between RPL and *HLA-G*0104* alleles in studies conducted on different ethnic populations worldwide.[22] *HLA-G*0104* allele is found at a frequency of 34.0% in the Korean population and other ethnic groups, such as

Table 2. Genotypic/allelic distribution of *HLA-G* Exon 3 gene in RM cases and healthy controls with respect to different clinic-pathological characteristics

Parameters	*HLA-G* exon G*0104 genotype	RPL cases		Control N 240 (%)	*p*-value
		Female N 200 (%)	Male N 100 (%)		
Specificity	G*0104N	26 (13)	7 (7)	2 (0.8)	Ref
	All but*0104N	174 (87)	93 (93)	238 (99.2)	<0.05
Allele	0104	52 (13)	14 (7)	4 (0.8)	Ref
	0103/0105	348 (87)	186 (93)	480 (99.2)	<0.05
Age					
<30	G*0104N	13 (12)	3 (9)	1 (0.8)	Ref
	All but*0104N	96 (88)	29 (91)	115 (99.2)	<0.05
≥30	G*0104N	13 (14)	4 (6)	1 (0.8)	Ref
	All but*0104N	78 (86)	64 (94)	123 (99.2)	<0.05
Miscarriage					
<3	G*0104N	11 (12)		2 (1.75)	Ref
	All but G*0104N	81 (88)		112 (99.2)	<0.05
≥3	G*0104N	15 (14)		0	Ref
	All but G*0104N	93 (86)		126 (100)	-
Family History					
Yes	G*0104N	0		0	Ref
	All but*0104N	32 (100)		41 (100)	1
No	G*0104N	25 (15)		2 (1.0)	Ref
	All but*0104N	145 (85)		197 (99)	<0.05
Consanguinity					
Yes	G*0104N	9 (16)		1 (3.3)	Ref
	All but*0104N	48 (84)		32 (96.7)	0.05
No	G*0104N	17 (12)		1 (0.09)	Ref
	All but*0104N	126 (88)		208 (99.1)	<0.05

Association done between females with RM and control group. RM, recurrent miscarriages; RPL, recurrent pregnancy loss.

Japan, China Han, and African Shona.[38,39] *HLA-G*0104* was also evaluated in POC samples in the present study, confirming that the frequency of transmission of this allele to the offspring was 6.7%. Thus, *HLA-G*0104* appears to be an important risk factor for RPL in our population compared to other populations.

Our study revealed that the exon 2 genotype of *HLA-G*, 0103:0103 was not detected in our population, neither in RPL patients nor in the control group. Furthermore, 60 POC samples genotyped for *HLA-G*0103* also lacked the 0103/0103 allele. The rarity or negligible occurrence of *HLA-G*0103* has been reported in several other populations in the world, with frequencies ranging from 0% to 2%.[28] In contrast to these reports, Park *et al*.[40] reported an *HLA-G*0103* frequency of ~24.2% among the Korean population, comprising the main *HLA-G* allele. Our region (Kashmir, North India) has different ethnic backgrounds and differs completely from the results reported by Park *et al.*[41] and Abbas *et al.*[36] Thus, *HLA-G*0103* seems to exhibit considerable genetic variation across different ethnic populations. Additionally, *HLA-G*0105N was not detected in either the case series or control group, indicating the absence of this allele in our population, a finding supported by Abbas *et al*.[36]

Furthermore, our study revealed *HLA-G*0104:0105N alleles implicated in comparable frequencies in both the RPL patient and controls, at 93% and 99.2%, respectively. These alleles were found to be significantly more prevalent in the controls compared to the reference *HLA-G*0104*, suggesting their non-pathogenic nature in RPL patients in our population. A similar scenario was observed by Matter *et al.,*[37] wherein although the two alleles *HLA-G*0104:0105N* were more frequent in the RPL group, but they did not achieve statistical significance.[35]

Inconsistencies have been observed among various studies due to the presence of different *HLA-G* genotypes and their pathogenic nature with respect to RPL. This variability could be attributed to several factors, including genetic variations unrelated to explored *HLA* alleles, polymorphic variants positioned elsewhere in the *HLA* gene, such as in the 5′, 3'UTR or intronic regions, and linkage disequilibrium to different polymorphic variants that lie in close proximity to the *HLA* locus. In summary, our findings sug-

Table 3. Genotypic/allelic distribution of *HLA-G* Exon 2 gene polymorphism in Recurrent Abortion cases and POC

Parameters	*HLA* exon 2 genotype	POC N = 60	Cases females N = 200 (%)	*p*-value
Over all genotype				
	G*0103	0	0	Ref
	All but G*0103	60 (100)	200 (100)	-
	G*0105N	0	0	Ref
	All but*0105N	60 (100)	200 (100)	
	G*0104N	4 (6.7)	26 (13.0)	Ref
	All but*0104N	56 (93.3)	173 (86.5)	0.03
Allele				
	0103	0	0	Ref
	0104/0105	120 (100)	400 (100)	-
	0105	0	0	Ref
	0104/0103	120 (100)	400 (100)	-
	0104	4 (3)	52 (13)	Ref
	0103/0105	116 (97)	348 (87)	0.003

POC, products of conception.

gest that certain *HLA-G* alleles have a substantial connection with an increased risk of RPL, and the combination of a few alleles can impact RPL patients.

Conclusions

In conclusion, the presence of the *HLA-G**0104 allele at a higher frequency in both partners strongly indicates a significant risk for RPL within our population. We further conclude that there is no role for *HLA-G* *0103 and *0105 in our population.

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Conflict of interest

All authors declare no conflict of interests.

Ethical statement

The study was approved by the local Institutional Ethics Committee SKIMS-Institutional Ethics Committee under protocol No. SKIMS Study ref: 81/2014. Patients were recruited after obtaining written informed consent. The procedures done involving human participants were as per the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments.

Data sharing statement

The data supporting the results of this study are available within the paper and it's Supplementary Information

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