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Association of rs1632944 and rs1632947 Polymorphisms of the *HLA-G* Gene Promoter with Recurrent Spontaneous Abortion among Azerbaijani Women from Northwest Iran

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ABSTRACT

Background: Genetic polymorphisms are considered potential causes of recurrent spontaneous abortions (RSA). This study aimed to determine the frequencies and association of rs1632944 and rs1632947 polymorphisms of the *HLA-G* gene promoter region with the RSA in women from northwest Iran.

Methods: This case-control study was conducted on 180 women referred to Madar Infertility Center and Al-Zahra Hospital in Tabriz. Ninety patients with a history of at least two RSA as the patient group and 90 women with at least one child and no history of abortion as the control group were included in the study. 5 ml peripheral blood was obtained from each person; genomic DNA was extracted, amplified by PCR, and genotyped with sequencing and ARMS methods. Data were analyzed using SPSS, Chi-square, and Fisher's exact tests.

Results: For rs1632944 polymorphism, frequencies of the AA, AG, and GG genotypes were 34.44%, 45.55%, and 20% for the control group and 48.88%, 46.66%, and 4.44% for the patient group, respectively. We found that the minor allele G is recessive against major allele A and might protect women against RSA. For rs1632947 polymorphism, frequencies of the GG, AG, and AA genotypes were 52.22%, 35.55%, and 12.23% for the patient group and 64.44%, 32.22%, and 3.33% for the control group, respectively. We found that the minor allele A is recessive against the major allele G and is associated with the RSA.

Conclusion: The results showed that the rs1632944 and rs1632947 polymorphisms of the HLAG-G gene promoter, might be related to RSA in women from Northwest Iran.

Keywords: Association study, HLA-G, Polymorphism, Recurrent spontaneous abortion, rs1632944, rs1632947

Introduction

Recurrent spontaneous abortion (RSA) -the occurrence of more than two consecutive miscarriages before the first half of pregnancy- is one of the main health problems of couples worldwide (1-3). The most common reasons for RSA are immunological, and a significant percentage of idiopathic abortions is caused by defective function of the mother's immune system on the fetus (4). One of the fundamental questions in pregnancy is how the mother's immune system does not identify and reject the fetus as a foreign entity. A look at the stages of embryo implantation shows that in all stages, the immune system plays a vital role in the acceptance of the embryo by the mother, and specific mechanisms defend the embryo against the mother's immune system and prevent the embryo from being damaged and rejected (5).

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For the acceptance of the fetus by the mother's immune system during pregnancy, many mechanisms function at the fetal-maternal interface. Human leukocyte antigen (HLA) class I antigens A and B and HLA class II are absent from fetal trophoblast cells, which assists in preventing allorecognition by B and T cells. In contrast, HLA-C, HLA-E, HLA-F, and HLA-G provide self-signals to control natural killer (NK) cell responses (6, 7). Non-classical HLAs such as HLA-G and its multiple isoforms are the most important antigens recognized by suppressor T cells (8). This antigen can force the lymphocytes in the maternal decidua cells to produce cytokines with positive and negative functions. Most couples suffering from RSA have some kind of alloimmune disorder that prevents the development of necessary immune reactions in the mother, which is favorable for the fetus's growth, survival, and continuation of the pregnancy (8, 9).

The *HLA-G* gene is located at the human chromosome 6p22.1 in the major histocompatibility complex (MHC) locus (10). The HLA-G pre-mRNA can generate seven distinct isoforms through alternative splicing, of which four are membrane-bound (HLA-G1, -G2, -G3, and -G4) and three are soluble (HLA-G5, -G6, and -G7) (11). It was reported that low levels of HLA-G were associated with the RSA (12, 13). In a successful pregnancy, the level of HLA-G in the mother's peripheral blood changes during pregnancy so that it increases in the first trimester, reaches its maximum level at the beginning of the third trimester, and decreases as the time of delivery approaches. Finding a decrease in HLA-G levels during the first weeks of pregnancy was associated with some complications, including RSA (14, 15). Changes in the blood levels of HLA-G might occur due to changes in its expression procedure. The most frequent causes of expression changes are polymorphisms, which affect the function of the corresponding promoter. Here, the polymer-phisms rs1632944 and rs1632947, located in the HLA-G gene's promoter region, were genotyped, and their association with the RSA was investigated.

Methods

Sampling

In this study, 90 patients who had a history of two or more recurrent spontaneous abortions and 90 people as control subjects who had no history of abortions were studied. The samples were obtained from people referred to Madar Infertility Center and Al-Zahra Hospital, Tabriz, Iran. The inclusion criteria for the patients were women who had experienced three or more spontaneous abortions (SAs), and the controls were women with at least one live birth and no SA. The exclusion criteria were women with recognized causes of RSA, such as metabolic, immunologic, environmental, thrombophilic, uterine anomalies, hormonal imbalances, chromosomal abnormalities, infections, endocrinological disorders, and diabetes. Only Azerbaijani women from northwest Iran were selected for this project. From each person, 5 ml of blood was taken intravenously, then the samples were transferred to the genetics laboratory and kept at 4°C.

Genomic DNA extraction

Genomic DNA was extracted from the blood using Miller's salting out method. The concentration and purity of the extracted DNA were determined by observing it on agarose gel and using a spectrophotometer. After DNA extraction, to ensure the extraction of genomic DNA, about 1µl of genomic DNA was electrophoresed on 1% agarose gel. After electrophoresis and staining with safe stain, the concentration and size of DNA molecules were determined by trans-illuminator. Primers were designed for each *rs* as shown in Table 1, and used for the amplification of the target sequence by PCR. The PCR products were run on 2% agarose gel, and their sizes were approved. The subjects' genotypes for rs1632944 were identified by sequencing (Microsynth Switzerland) and Chromas software.

Table 1. Primers used for amplification and genotype identification of rs1632944 and rs1632947 polymorphisms

	Sequence5'-3'	BC	Tm(°C)
Rs1632944			
Forward	TCGCTGGGTGTTCTTTGC	18	56
Reverse	GAGATAGAATAGAGACCAGTTTGC	24	59
rs1632947			
Forward outer	ACTCACACGGAAACTTAGG	19	55
Reverse outer	GCGTTCTGTCTCAGTGTC	18	56
Forward inner	CATTCAGGGGTTACCAAGG	19	57
Reverse inner	TTTATTAACTATAGTGGGTAGCATACA	27	61

The Tetra primer ARMS-PCR method was used for genotyping the rs1632947 polymorphism. The primers are listed in Table 1. The target region of the HLA-G gene was amplified using the Tetra-ARMS protocol. The PCR products were electrophoresed on 2% agarose gel to identify the **subjects' genotypes.**

Statistical Analysis

GraphPad Prism v.8 software was used to perform statistical analysis. Fisher's exact test was used to determine differences between groups. Hardy-Weinberg equilibrium (HWE) was also analyzed by a Chi-square (χ^2) test. Values of $p < 0.05$ were considered as significant. Based on the literature, the frequency of RSA (5%), and a significant level of 0.05, the minimum sample size needed for the study was calculated to be 76.

Ethical Approval

Informed consent was obtained from the participants in the study, and the research protocol was approved by the Medical Ethics Committee of the University of Tabriz (IR.TABRIZU.REC.1398.029).

Results

Genotype and Allele frequencies of the rs1632944 polymorphism

Genotypes of the patient and control groups for rs1632944 polymorphism were identified by PCR sequencing (Figure 1). The Hardy-Weinberg equilibrium analysis of the patient, control, and total population revealed that the population is in the equilibrium for rs1632944 polymorphism (Table 2). Frequencies of the AA, AG, and GG genotypes were 34.44%, 45.55%, and 20% for the control group and 48.88%, 46.66%, and 4.44% for the patient group, respectively. Both AA and GG homozygotes frequencies were significantly different between the patient and control groups ($p<0.05$), but the difference between the AG heterozygotes was not statistically significant ($p>0.05$) (Table 3). Frequencies of the A (reference allele) and G (alternative allele) were 72.22% and 27.78%, respectively, for the patients' group and 57.22% and 42.77% for the control group. Statistical analysis showed that the frequency of allele G significantly differed between the two groups ($p = 0.004$). As outlined in Table 3, different allelic interactions, including dominant/recessive, co-dominance, and over-dominance inheritance models, were also analyzed. Results showed that the allele G is recessive against the allele A.

Genotype and Allele frequencies of the rs1632947 polymorphism

Tetra primers ARMS-PCR was applied for genotyping of the rs1632947 polymorphism of the

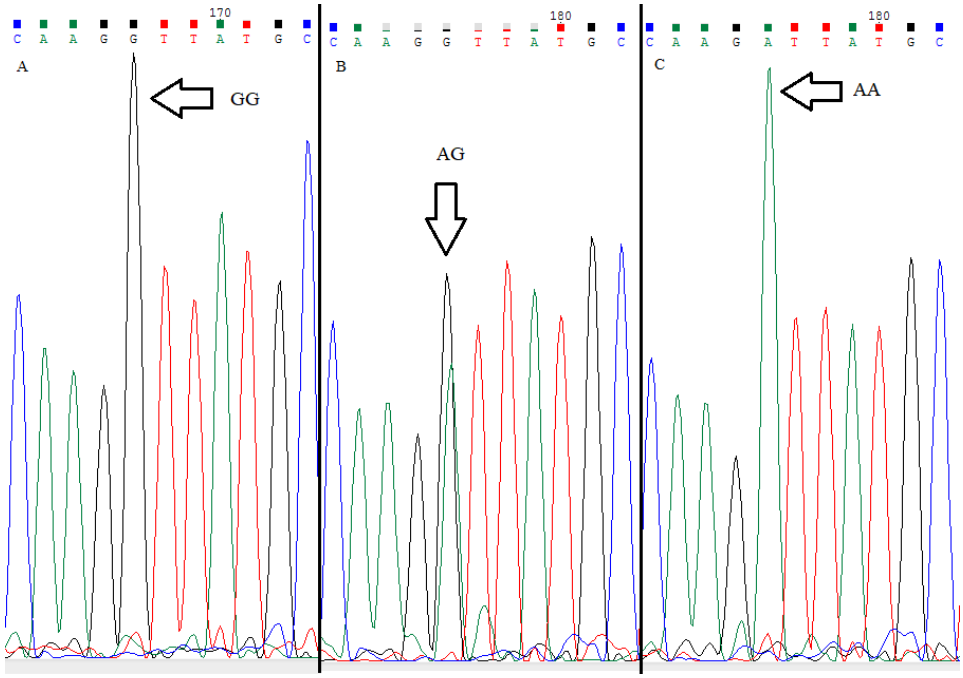

Figure 1. Sample chromatograms indicating different genotypes of rs1632944 polymorphism; A) homozygote GG, B) heterozygote AG and C) homozygote AA

Table 2. Hardy-Weinberg Equilibrium (HWE) test for genotypic frequencies of rs1632944

Genotype	Patient(n)	HWE		Control(n)	HWE	
		χ^2	P		χ^2	P
		2.38	0.3		0.43	0.8
AA	44			31		
AG	42			41		
GG	4			18		

HWE; Hardy-Weinberg Equilibrium, p=p value, χ^2; chi

patient and control groups (Figure 2). The Hardy-Weinberg equilibrium analysis of the patient, control, and total population revealed that the population is in the equilibrium for rs1632947 polymorphism (Table 4). Frequencies of the GG, AG, and AA genotypes were 52.22%, 35.55%, and 12.22% for the patient group and 64.44%, 32.22%, and 3.33% for the control group, respectively. The allelic frequencies of this polymorphism for G (reference allele) and A (alternative allele) were 70% and 30% for the patient group and 80.55% and 19.44% for the control group, respectively. Furthermore, different allelic interactions, including dominant/recessive, co-dominance, and over-dominance inheritance models were also analyzed. We found that the allele A is recessive against the allele G (Table 5).

Table 3. Genotypic and allelic distribution of rs1632944 HLA-G gene polymorphism and analysis of different allelic interactions

Genotypes	Patient (n=90)	Control (n=90)	Odds ratio	P	CI (95%CI)
AA	44	31	1.865	0.0442*	1.04 to 3.27
AG	42	41	1.041	>0.9999	0.59 to 1.83
GG	4	18	0.17	0.001***	0.05 to 0.5
Allele					
A	130(72.22%)	103(57.22%)	1.94	0.002*	1.25 to 3.02
G	50(27.78%)	77(42.77%)	1	-	-
Co-Dominant					
AG	42(46.66%)	41(45.55%)	4.6	0.005**	1.44 to 14.79
AA	44(48.88%)	31(34.44%)	6.39	0.001***	1.97 to 20.72
GG	4(4.44%)	18(20%)	1	-	-
Recessive					
AA	44(48.88%)	31(34.44%)	6.39	0.001***	1.97 to 20.72
AG+GG	46(51.12%)	59(64.63%)	1	-	-
Dominant					
GG	4(4.45%)	18(20%)	1	-	-
AA+AG	86(95.55%)	72(80 %)	4.72	0.003**	1.53 to 14.53
Over-Dominant					
AG	42(45.83%)	41(45.66%)	1.05)	0.50	0.58 to 1.88
AA+GG	48(54.16%)	49(54.44%)	1	-	-

CI: confidence Interval. p= p value *: Significance less than 0.05. **: Less than 0.01. ***: Significance less than 0.001

Table 4. Hardy-Weinberg Equilibrium (HWE) test for genotypic frequencies of rs1632947

Genotype	Patient (n)	HWE		Control (n)	HWE	
		χ^2	P		χ^2	P
		2.12	0.34		0.07	0.96
GG	47			58		
AG	32			29		
AA	11			3		

HWE; Hardy-Weinberg Equilibrium, p=p value, χ^2; chi square

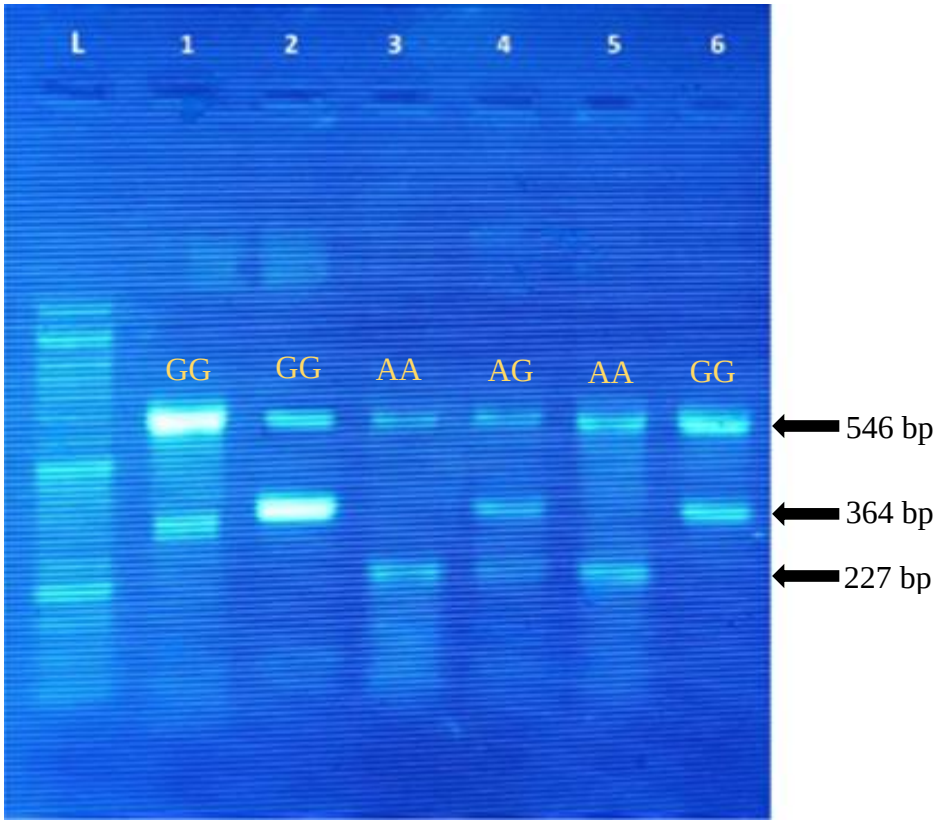

Figure 2. Agarose gel electrophoresis of the tetra-primer ARMS- PCR products. Lane L indicates the DNA size marker, and lanes 1 to 6 indicate different genotypes identified by the tetra-primer ARMS-PCR genotyping method. The Band 564 bp (internal control) is the product of amplification by outer forward and reverse primers. The bands 364 and 227 are products of the allele-specific inner primers and outer reverse primers. Amplification by a G allele-specific primer results in a 364 bp PCR product, while amplification by an allele-specific primer produces a 227 bp PCR product. Different genotypes have been shown in the gel

Table 5. Genotypic and allelic distribution of rs1632947 polymorphism and analysis of different allelic interactions

Genotype	Patient (n=90)	Control (n=90)	Odds ratio	P	CI (95%CI)
GG	47	58	0.603	0.065	0.332 to 1.096
AG	32	29	1.161	0.376	0.626 to 2.153
AA	11	3	4.38	0.024*	1.087 to 15.002
Allele					
G	126(70%)	145(80.55%)	1	-	-
A	54(30%)	35(19.44%)	1.776	0.014*	1.09 to 2.892
Co-Dominant					
GG	47(52.22%)	58(64.44%)	1	-	-
AG	32(35.55%)	29(32.22%)	1.36	0.21	0.72 to 2.56
AA	11(12.23%)	3(3.33%)	7.33	0.002**	1.9 to 28.35
Dominant					
GG	47(52.22%)	58(64.44%)	1	-	-
AA+AG	43(47.78%)	32(35.66%)	1.66	0.065	0.91 to 3.01
Recessive					
AA	11(12.23%)	3(3.33%)	4.04	0.024*	1.09 to 15.0
GG+AG	79(87.77%)	87(96.67%)	1	-	-
Over-Dominant					
AG	32(35.55%)	29(32.22%)	1.16	0.38	0.63 to 2.15
GG+AA	58(64.45%)	61(67.77%)	1	-	-

CI: confidence Interval. P= P value. *: Significance less than 0.05. **: Significance less than 0.01

Table 6. Allele frequency of rs1632944 polymorphism in global and different subpopulations based on the ALFA project.

Population	Group	Sample Size	Ref Allele	Alt Allele
Total	Global	19190	G=0.53090	A=0.46910
European	Sub	14286	G=0.54543	A=0.45457
African	Sub	2970	G=0.4848	A=0.5152
African Others	Sub	114	G=0.439	A=0.561
African American	Sub	2856	G=0.4867	A=0.5133
Asian	Sub	116	G=0.302	A=0.698
East Asian	Sub	88	G=0.33	A=0.67
Other Asian	Sub	28	G=0.21	A=0.79
Latin American 1	Sub	154	G=0.539	A=0.461
Latin American 2	Sub	616	G=0.489	A=0.511
South Asian	Sub	98	G=0.33	A=0.67
Other	Sub	950	G=0.532	A=0.468

Discussion

HLA-G inhibits the function of some immune cells, such as NK cells and T CD8+ lymphocytes (16), and plays a critical role in the acceptance of the fetus by the mother's immune system during pregnancy (17). The HLA-G coding is relatively conserved and shows few polymorphisms, but its regulatory regions carry more polymorphisms and are more variable. These regulatory regions have target sites for several transcriptional and post-transcriptional regulatory elements (18), and some polymorphisms in these segments have been associated with different expression profiles (19). The level of HLA-G expression in pathological and normal conditions varies between individuals, indicating that the HLA-G gene promoter polymorphisms impact its expression profile (20).

In the present study, we genotyped rs16329444 and rs1632947 polymorphisms in the promoter region of the *HLA-G* gene to assess their association with RSA in Azerbaijani women living in northwest Iran. We found that the minor Allele G and the genotype GG in the rs1632944 polymorphism have a protective effect so that it might protect women against RSA. In contrast, the genotype AA and Allele A frequencies were significantly higher in the patient group than in the control, indicating a positive association with the RSA. These results align with the finding of Berger et al. (21), who examined the association of this polymorphism with the RSA in Caucasian women. However, heterozygote genotype AG showed no significant difference between the patient and control groups. Based on the ALFA (Allele Frequency Aggregator) project (22), in the global population, Allele G has a frequency of 0.53090, which is the major Allele. However, as outlined in Table 6, the major Allele depends on the sub-populations. The major Allele in Asian and sub-Asian populations, in contrast to the other populations, such as the European population, is the Allele A, which is consistent with our results. However, in a recent study by Merdas et al. (23) in an Iraqi population, Allele G was reported as the major Allele, and they did not find any association with the RSA in the studied population. Their result was different from our findings. Ober et al. (24) conducted a 15-year prospective study of pregnancy outcomes on the Hutterites of the South Dakota population, which were descendants of only 64 Hutterite founders. They studied associations of 16 polymorphisms, including rs16329444. The frequency of Allele A was 0.36 and showed no association with the RSA in the Hutterites population. However, they found a relation between RSA and another polymorphism located at the -725 site.

To understand the allelic interaction between the major allele A and minor allele G and reveal their inheritance model, we analyzed the data based on the dominance/recessive, co-dominance, and over-dominance inheritance models. The results indicated that the major allele A is dominant against the protective allele G, meaning that the allele A might affect RSA in its homozygote situation.

The other polymorphism we analyzed in this study was rs1632947, located in the HLA-G gene's promoter region. The population was in Hardy-Weinberg equilibrium regarding this polymorphism. The frequency of minor allele A was 30% and 19.44% in the patient and control groups, respectively, indicating a significant difference between the two groups (p=0.027). Also, we found a significant difference in the AA genotype frequency between the patient and control groups (p=0.024). Besides, analyzing the different models of allelic interactions revealed

that minor allele A is recessive against major allele G, which could affect the RSA in the homozygote AA women. Berger et al. (21) examined the association of rs1632947 polymorphism with the RSA in Caucasian women. They found a positive association between the minor allele A and RSA (p=0.024, OR=1.42), consistent with our findings. The odds ratio in our study was 4.38, indicating a higher association power with the RSA in the study population.

In contrast to these results, Ober et al. (24) did not detect any significant association between this polymorphism and RSA in the Hutterites population. Also, in the study by Merdas et al. (23) on Iraqi women, there was no significant difference between patient and control groups in the allele A frequency, indicating no association between this polymorphism and RSA. In 2019, Nowak et al. (25) in Poland examined a group of women with a history of recurrent miscarriage as a patient group and women without a history of recurrent miscarriage as a control group. They found a significant association of rs1632947 polymorphism with recurrent miscarriage. There are other polymorphisms in the regulatory regions of the *HLA-G* gene that researchers in different populations have reported as being associated with the RSA. Among these are rs1736933 (21, 26), rs2735022 (24, 27), rs2249863 (21, 23, 28), rs1632946, rs1736932, rs1632943, rs1631950 (21), rs123334 (24, 28) polymorphisms.

To have a complete understanding of the effect of the *HLA-G* gene on the RSA, studying the gene's polymorphisms and their haplotypes is needed. Also, a study on a larger population can provide more accurate results. Based on the present and other studies that link RSA to the *HLA-G* gene polymorphisms, we suggest further analysis of the other *HLA-G* polymorphisms and their possible haplotypes in Northwest Iran and other ethnic groups of Iran.

Conclusion

Results of the present study indicated that the allele A and genotype AA of the rs1632944 and the allele A and genotype AA of the rs1632947 polymorphisms are associated with the RSA in the women Northwest Iran population.

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None.

Conflicts of interest

There was no conflict of interest.

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