Short Communication

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Association Analysis of the HLA-G Upstream Polymorphism Rs1736933 with the Recurrent Spontaneous Abortion

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ABSTRACT

BACKGROUND AND OBJECTIVE: Studies have suggested that HLA-G is involved in protecting the fetus against the mother's immune system, and upstream polymorphisms of this gene may influence pregnancy success by effecting its expression. The aim of this study was to evaluate the association of upstream HLA-G gene polymorphism, rs1736933, with recurrent spontaneous abortion in East Azerbaijan women population.

METHODS: This case-control study was performed on 80 women with at least one child and without any history of abortion, and 100 women with at least two recurrent spontaneous abortions which were diagnosed by gynecologist. 2 ml of blood was obtained from the participants and after DNA extraction, a segment of the HLA-G gene promoter was amplified by PCR and analyzed by agarose gel electrophoresis. PCR products were sequenced and genotyped. Allelic and genotypic frequencies of patient and control groups were compared.

FINDINGS: Frequency of AA, CA, CC genotypes in the patient group were 8(8%), 37(37%), 55(55%) and in the control group were 16(20%), 33(41.25%), 31(38.75%) respectively. Comparison of genotypic frequencies between the two groups showed that CC homozygous genotype was associated with recurrent abortion (p= 0.015). Statistical analysis also showed a significant difference between the allelic frequencies of the two groups (p= 0.019).

CONCLUSION: The results of this study showed that the upstream HLA-G gene polymorphism, rs1736933, is associated with the recurrent abortion in East Azerbaijan women population.

KEY WORDS: *Recurrent Abortion, Polymorphism, HLA-G, Association Studies.*

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Introduction

Recurrent abortion means at least two to three spontaneous abortions in the 20th week of pregnancy or the birth of a fetus weighing less than 500 grams (1). Its prevalence in couples who intend to have children is estimated at 1 to 3% (2). Early identification of the potential risk of abortion and systemic control has a beneficial effect on increasing the live birth rate in couples with a history of recurrent abortion (3). Although this disease has several causes including cytogenetic disorders, uterine deformity, hereditary thrombophilia, autoimmunity, sperm problems and environmental factors, but the cause remains unclear in most patients (4). On the other hand, studies show that recurrent abortion is associated with polymorphism of genes involved in pregnancy (5, 6).

Among the genes that have been shown to be associated with this disease are TNF, IL6, VEG, AR, eNOS, HLA-G (7). In addition, next-generation sequencing technology has greatly contributed to the genetic etiology of the disease (8). HLA-G is expressed on fetal placental cells (9) and plays a vital role in the implantation, development and differentiation of trophoblasts and their attachment to the endometrium of the uterus, as well as in the process of angiogenesis to provide sufficient oxygen to the fetus (10). This molecule induces tolerance in the maternal immune system by inhibiting NK (Natural killer cell) cells, causing the fetus to be tolerated and preventing its rejection by the maternal immune system (11). Also, changes in serum concentration and expression in placental tissue of individuals with recurrent abortion have been reported (12-14).

Exclusive expression of HLA-G at the mother-fetus border, unlike classical HLA antigens expressed in all parts of the body, indicates that HLA-G has a special role in these areas (15). Reports indicate that mutations in the HLA-G gene or its upstream polymorphisms may affect pregnancy success by modulating HLA-G gene expression (16, 17). Numerous polymorphisms have been identified in the 3´ UTR region (18) as well as the 5´ UTR region and the promoter region (19) of this gene that are associated with recurrent abortion. The rs 1736933 polymorphism is located in the promoter region of the HLA-G gene and is also known as A> C-486 (20). This polymorphism affects the binding of heat shock factor to the heat shock element and leads to early differences in HLA-G gene expression (21). Hviid et al. showed that HLA-G acts as a ligand for NK cell receptors and is able to inhibit the function of NK cells, and if this activity is not controlled can potentially harm the fetus (22). On the other hand, Ober et al. showed a decrease in HLA-G serum levels in patients with spontaneous recurrent abortion and the association of -486 polymorphism with that disease (23). Due to the fact that the allelic frequency of polymorphisms in different populations depends on the genetic background of those populations, so the polymorphism frequency of rs1736933, the HLA-G upstream gene in the female population of East Azerbaijan province may be different from other populations. Therefore, the aim of this study was to investigate the genotypic and allelic distribution of the polymorphism as well as its association with recurrent abortion in the female population of East Azerbaijan province.

Methods

This case-control study was approved by the Ethics Committee of Tabriz University of Medical Sciences with the code TBZMED.REC.1398.208 and was performed after obtaining informed consent from patients and control, on 100 women with spontaneous recurrent abortion and 80 women without abortion as control group that was selected from the patients referred to Al-Zahra Hospital and Tabriz Mother Clinic by a gynecologist.

Subjects with at least two recurrent abortions for no reason were included to study and if they had TORCH syndrome (toxoplasmosis, rubella, cytomegalo virus, herpes virus), chromosomal problems, hormonal disorders, and uterine anatomical disorders were excluded from the study. Inclusion criteria for control group were having at least one child with a normal pregnancy and exclusion criteria were having a history of abortion or using assisted reproductive technology.

Miller salt method was used to extract genomic DNA from blood samples with some modifications (24). The quantity and quality of DNA extracted were evaluated by spectrophotometer and agarose gel. Gene Runner and Primer-Blast software were used to design the primers. Amplification of fragment containing 486-polymorphism was performed by PCR by forward primer (3'-GACTCACACGGAAACTTAGG-5') and reverse primer (5'-ACACAGGTTAGGAGAAGGAG-3'). The PCR process in a thermocycler (Peqlab-pecStar, Jermany) involves initial denaturation, a five-minute cycle at 95 ° C, followed by 40 cycles containing 30 seconds of denaturation at 95 ° C, 30 seconds of anneling at 58 ° C, 30 seconds of amplification at 72 ° C and finally for 5 minutes at 72 ° C. Finally, PCR

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products were sent to the Swiss company Microscience for sequencing. Sequences were checked with Chromas software and genotypes were determined. The obtained data were analyzed using SPSS software and x2 test and p≤0.05 was considered statistically significant.

Results

Genotyping of control of patient groups: To determine the genotype of individuals for rs1736933 polymorphism 684 bp fragments from the promoter region of HLA-G gene was amplified by PCR. Figure 1 shows the electrophoresis of several PCR products.

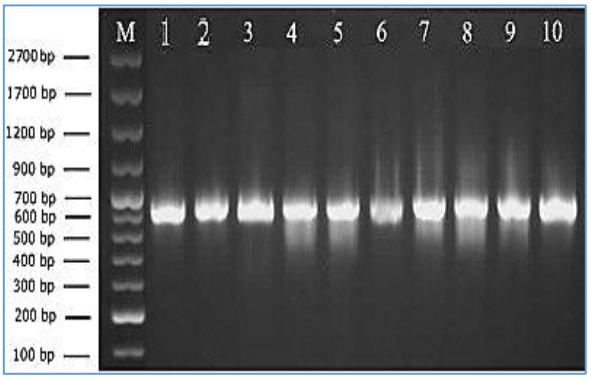

Figure 1. Electrophoresis of PCR products: M represents DNA marker and 1-10 columns are PCR products of different samples

In the next step, PCR products were sequenced and the individuals' genotypes were read from the resulting chromatograms. Figure 2 shows examples of chromatograms containing different genotypes of the rs1736933 polymorphism site.

Genotypic distribution of polymorphism - 486 in the patient group and their relationship with recurrent abortion: In this study, among patients, 8 patients were homozygous (AA), 37 patients were heterozygous (CA) and 55 patients were diagnosed as homozygous (CC). Among the control group, 16 individuals were homozygous (AA), 33 individuals were heterozygous (CA) and 31 individuals were homozygous (CC) (Table 1). Comparison of genotypic frequencies of patient and control groups showed that CC genotype was significantly different between the two groups (p = 0.015) and the resulting of Odds Ratio showed that CC genotype was associated with recurrent abortion (OR=1.932).

Allelic frequencies of polymorphism (-486) of patient and control groups and their relationship with recurrent abortion: Frequency of allele A (normal allele) for the patient and control group was 26.5% and 40.62% respectively and frequency of C allele for the patient group and Control was calculated to be 73.5% and 59.38%, respectively. Comparison of allelic frequencies between patient and control groups showed a significant difference between C allele between patient and control groups (p = 0.019) and the amount of Odds Ratio showed that C allele was associated with recurrent abortion in the study population. (OR = 1.897) (Table 1).

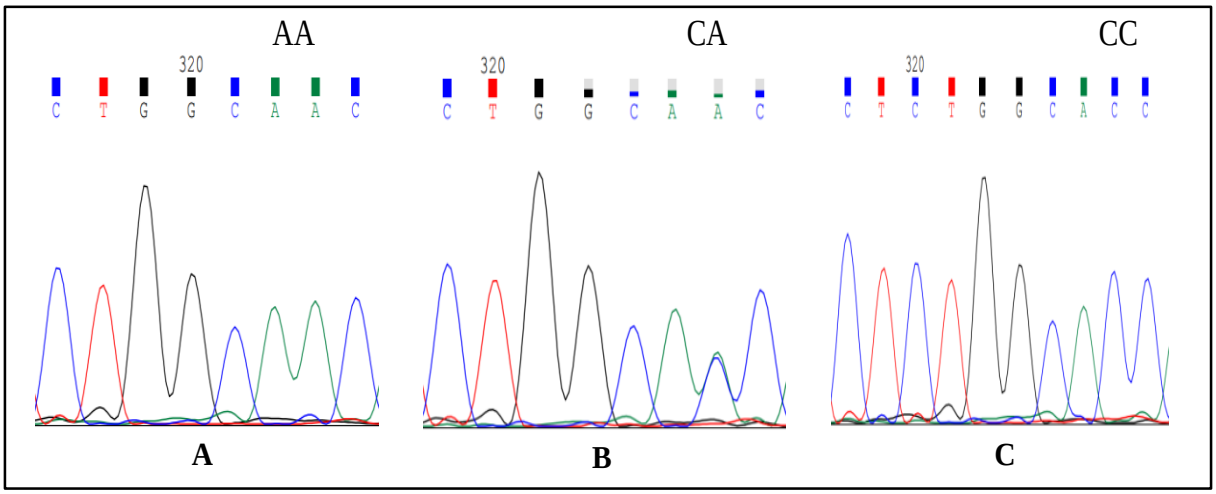

Figure 2. Examples of chromatograms obtained from sequencing PCR products related to HLA-G gene:
A) Chromatogram of individual with homozygous AA genotype
B) Chromatogram of individual with heterozygous genotype CA
C) Chromatogram of individual with homozygous genotype CC

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Table 1. Genotypic and allelic frequencies of polymorphism (-486) of HLA-G gene in patients with recurrent abortion and control group

variables	Patient (n = 100) Number(%)	Control (n = 100) Number(%)	Odds Ratio	(CI 95%)	P-value
Genotype					
AA	8(8)	16(20)	0.348	0.132-0.891	0.012
CA	37(37)	33(41.75)	0.836	0.455-1.537	0.319
CC	55(55)	31(38.75)	1.932	1.059-3.533	0.015
Allele					
A	53(26.5)	65(40.62)	0.577	0.777-0.999	0.017
C	147(73.5)	95(59.38)	1.897	1.001-3.605	0.019

Discussion

In this study, comparison of genotypic frequencies between patient and control groups showed that homozygous CC genotype is associated with recurrent abortion (OR = 1.932 and p = 0.015). Also, the frequency of C allele in the patient group was significantly different from the control group (OR = 1.897 and p = 0.019), which indicates that it is associated with recurrent abortion in the studied population. In a normal pregnancy, several factors are involved (25), including cytokines, hormones, growth factors, and angiogenic factors (7). Researchers in different countries have studied most of the genes involved in recurrent abortion, such as genes involved in immune tolerance, inflammation, maternal metabolism changes, thrombophilia, as well as genes of chromosome structural defects (26). One of these genes is the HLA-G gene, which has multiple polymorphisms, and these polymorphisms may potentially affect all the features and biological functions of HLA-G (20). 3´UTR regions (18) and HLA-G promoter (19) have different polymorphisms that can affect the expression and HLA-G protein level, including A> C-486 polymorphism (27). A total of 32 SNPs were identified according to Berger et al.'s study of HLA-G (28). They also calculated the frequency of C allele for the 486-position in the patient and control groups as 0.500 and 0.418, respectively, and showed that the C allele with OR = 1.41 and CI = 1.073--.851 and p=0.004, shows a positive association with recurrent abortion (29). Agrawal et al. studied a total of 27 single nucleotide polymorphisms and showed that the C allele at the polymorphism site of -486 was associated with recurrent abortion (p = 0.019-048 and OR = 2.35), and that the C allele was an risk allele (30). Hviid et al. also identified 11 polymorphisms, including -486 polymorphisms, in their study of 61 Caucasian couples. They showed that polymorphisms in the 5´URR (Upstream Regulatory Region) of the HLA-G gene could affect the secretion of interleukin-10. Also, polymorphism -486, which is located in the promoter of HLA-G gene, showed a significant relationship with the concentration of interleukin-10. Their study showed that polymorphism -486 has functional significance (31). The results of the present study are consistent with the results of Berger et al (29). However, the frequency of C alleles in our study is lower than that of Berger et al. Also, in comparison with the results of Agrawal et al. (30), a greater allelic difference is observed than the present study. These differences are due to the genetic background of the studied populations. The results of this study showed that C allele and CC genotype in polymorphism -486 in the population of Azeri women in East Azerbaijan province is positively associated with recurrent abortion and examining the genotype of individuals in relation to this position may be helpful for gynecologists in making decisions and providing health care services.

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