Fetal HLA-G alleles and their effect on miscarriage

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Abstract

Background. Immunossupression at the feto-maternal interface is crucial for a successful pregnancy outcome. Human leukocyte antigen–G (HLA–G) seems to be a major contributor to fetal tolerance. The HLA–G expression is seen in cytotrophoblasts and in maternal blood. Fetal HLA–G acts on decidual antigen-presenting cells (APCs), natural killers (NKs) and T cells. Recent findings revealed that defects in placentation and their consequences are associated with maternal HLA–G variants and their expression levels.

Objectives. The objective of this article is to investigate the relationship between fetal HLA–G alleles and miscarriage, which has not been investigated to date.

Material and methods. The present study includes 204 recurrent miscarriage (RM) cases who were admitted to our clinic between 2012 and 2016. Twenty-eight miscarriage products without maternal cell contamination and any known pathology were analyzed by HLA–G typing. In addition, 3’ untranslated region (UTR) 14-base pair (bp) insertion/deletion polymorphism was also investigated by Sanger sequencing.

Results. For our population, the most frequent HLA–G type was G*01:01, both in the study group (30.3%) and in the control group (47%). The study revealed that the G*01:04 allele was significantly associated with miscarriage (p = 0.007). The 3’ UTR 14bp deletion was more frequent in the miscarriage group, but there was no significant correlation.

Conclusions. HLA–G alleles seem to be related with miscarriage and should be considered in RM cases.

Key words: miscarriage, human leukocyte antigen–G, G*01:04, 3’ untranslated region polymorphism
Introduction

Human leukocyte antigen-G (HLA-G) is an atypical HLA (Class Ib) molecule and is mainly expressed in immune-privileged sites of the body. There are accumulating reports on the possible involvement of HLA-G in cancer, transplantation, allergies, and autoimmune disorders. The role of HLA-G in the maintenance of maternal tolerance and in reproduction has been also widely studied. The expression of HLA-G variants, including membrane-bound (HLA-G1), soluble (HLA-G3 and 4), and membrane-bound (HLA-G5 to -G7) isoforms, is highly polymorphic and only have a limited number of alleles and membrane-bound forms. The expression of HLA-G was first described in the placenta as the conventional β2m-linked, membrane-bound form. However, studies reveal that HLA-G has 7 splice variants, including 4 membrane-bound (HLA-G1 to -G4) and 3 soluble isoforms (HLA-G5 to -G7). Furthermore, membrane-bound HLA-G1 can be shed and released as soluble HLA-G1. About 50 HLA-G alleles and 16 proteins have been reported to date.

HLA-G has 7 introns and 8 exons. Exon 1 is related to a signal peptide. Exons 2, 3, and 4 encode the extracellular domains: α1, α2 and α3, respectively. Intron 4 contains a stop sequence and yields soluble G5 and G6 isoforms. The transmembrane domain is encoded by exon 5. Exons 6 and 7 encode the intracellular domain.

The polymorphisms of HLA-G have also been widely studied. The HLA-G gene has 14-base pair (bp) insertion/deletion (ins/del) polymorphism in 3′ untranslated region (UTR), which influences messenger RNA (mRNA) size and stability. An inserted 14bp sequence probably acts as a cryptic splice site and causes a 92bp deletion in 3′ UTR. As a result, HLA-G mRNA stability is increased.

Recent studies have focused on the functions of HLA-G isoforms, HLA-G dimers and β2-microglobulin (β2m) association. The disulfide-linked homodimer of β2m-associated HLA-G is found to be the major fraction expressed by trophoblast cells. LIRB1 has been proposed as the principal ligand for the HLA-G dimer, which is expressed on decidual APCs, NKs and T lymphocytes. Interactions with LIRB2, CD160, KIR2DL4, and other receptors have also been reported.

HLA-G alleles have been investigated in women with a history of miscarriage and in their partners, but not in conceptus material to date. One of the reasons for that is the difficulty in obtaining maternal-cell-free fetal tissues and the increased failure rates for cell culture and polymerase chain reaction (PCR). In addition to these factors, many of the miscarriage samples contain chromosomal anomalies which should be excluded from the study groups. The determination of parental HLA-G alleles, as in the current literature, just leads to indirect estimations about the fetal HLA-G status. Definite fetal HLA-G genotyping should be the preferred approach instead of parental genotyping, because HLA-G is expressed by fetal trophoblasts. In this study, we aimed to find an association between fetal HLA-G type, HLA-G 3′ UTR 14bp ins/del polymorphism and miscarriage, and to determine the most common HLA-G alleles in our population.

Material and methods

Study and control groups

The study group consisted of 28 cases out of 204 patients referred to our center between 2012 and 2016 who had at least 2 miscarriages (range: 2–4). All of the mothers were investigated for hereditary thrombophilia. Miscarriage samples were karyotyped and analyzed by quantitative fluorescent polymerase chain reaction (QF-PCR) for aneuploidies of chromosomes 13, 15, 16, 18, 21, and 22, and sex chromosomes. The short tandem repeat (STR) markers of a QF-PCR kit were also used to detect maternal cell contamination. At least 10 informative STR markers were needed to exclude maternal cell contamination. The samples with 46,XY karyotype or 46,XX karyotype with no maternal contamination were included. Miscarriage materials with chromosomal anomalies, culture failure or PCR failure were excluded. The mothers with thrombophilic mutations, a history of in vitro fertilization (IVF) or any known obstetric reason for miscarriage, such as uterine malformations, antiphospholipid syndrome or hormone disorders, were also excluded. Fetuses with anencephaly, increased nuchal translucency, anhydramnios, early rupture of membranes, or other major malformations on fetal ultrasonography were excluded. The mean age...
of mothers was 30.3 years and the majority of pregnancy losses occurred during the 1st trimester (gestational weeks 5–15). Twenty-one healthy individuals representing successful deliveries were used as controls for HLA-G types determined by the sequencing of exons 2, 3 and 4. For 3' UTR polymorphism, 101 healthy individuals were used as controls. The project received the permission of the local Tepecik Training and Research Hospital ethics committee.

**Routine genetic workup of miscarriage**

The routine genetic investigation of miscarriage cases included a conventional cytogenetic analysis of the miscarriage sample by GTG banding (550 bands) and a QF-PCR analysis with 26 STR markers (Compact v3 QF-PCR; Devyser, Stockholm, Sweden). Hereditary thrombophilia mutations (Factor V Leiden and Factor II G20210A) were investigated by real-time PCR (FV R2 [H1299R] QLP 3.0; Prothrombin QLP 3.0, Iontek, Istanbul, Turkey) from maternal blood.

**HLA-G typing**

Exons 2, 3 and 4 were sequenced for HLA-G typing. Commercial primer/oligonucleotide sets dedicated for high-resolution HLA sequencing-based typing (SBT) for the identification of HLA alleles were used according to the manufacturer’s protocols (SBTexcelerator® HLA Kits; GenDx, Utrecht, the Netherlands).

**HLA-G 3' UTR 14bp ins/del polymorphism (rs371194629)**

The polymorphism was investigated by Sanger sequencing. The PCR primers were F: 5'-TGT GAA ACA GCT GCC CTG TGT-3' and R: 5'-GTC TTC CAT TTA TTT TGT-3'. The PCR conditions were as follows: the first denaturation was at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and elongation at 72°C for 45 s; and the last elongation was at 72°C for 7 min. The PCR products were purified as follows: 5 µL of PCR products were treated with 2 µL of ExoSAP-IT enzyme (USB; Affymetrix, Santa Clara, USA) at 37°C for 30 min and at 85°C for 15 min. Sequence PCR (cycle sequencing) was done using a reverse PCR primer (5 pmol) and a BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Lifetechnologies, Waltham, USA). The sequence PCR conditions were as follows: at 96°C for 10 s, at 50°C for 5 s and at 60°C for 4 min; the cycle was repeated 25 times. The products of sequence PCR were purified (the 2nd purification) by spin colon (ZR DNA Sequencing Clean-up Kit™, Zymo Research, Irvine, USA). Sanger sequencing was performed by capillary electrophoresis after 5 min of denaturation (3500 Genetic Analyzer; Lifetechnologies). The obtained sequences were analyzed using SeqScape® software v. 3.0 (Applied Biosystems by Life Technologies, Carlsbad, USA).

**Statistical analysis**

Allelic and genotypic frequencies were determined from the observed genotype counts, and the expectations of the Hardy-Weinberg equilibrium were evaluated by χ² analysis. The χ² test was used for comparisons between allelic and genotypic frequencies. Statistical analysis was done using SPSS v. 13 statistical software (SPSS Inc., Chicago, USA). A p-value <0.05 was considered statistically significant.

**Results**

In all groups, there were 10 HLA-G types, coded for 4 distinct proteins, and their combinations gave rise to 8 distinct genotypes. The most frequent HLA-G type was G*01:01 both in the study group (30.3%) and in the control group (47%). The findings are summarized in Table 1. G*01:01/*01:01 was the most frequent genotype for the study and control groups: its frequency was 35.7% in the study group and 48% among the controls. The genotypes G*01:04/*01:04, G*01:03/*01:04 and G*01:03/*01:06 were found only in the study group, and the G*01:06/*01:06 genotype was found only in the control group (Table 2). There was a significant association between the HLA-G allele G*01:04 and miscarriage (p = 0.007).

The results of 3’ UTR polymorphism are presented in Table 3. The frequency distribution of alleles was in Hardy-Weinberg equilibrium. In the miscarriage samples, the most frequent allele was the 14bp deletion (57%) and the most frequent genotype was homozygous deletion (39%). Homozygous deletion was approx. 2 times more frequent.
in the miscarriage group than in the controls. In the control group, the most frequent allele was the allele with insertion (54%), and heterozygosity was the most frequent genotype (53%).

Table 2. Comparison of HLA-G genotype frequencies in study and control groups

<table>
<thead>
<tr>
<th>HLA-G genotypes**</th>
<th>Frequencies in miscarriage samples, % (n)</th>
<th>Frequencies in control samples, % (n)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>*01:01/*01:01</td>
<td>35.7 (10/28)</td>
<td>48 (10/21)</td>
<td></td>
</tr>
<tr>
<td>*01:01/*01:04</td>
<td>25 (7/28)</td>
<td>9.5 (2/21)</td>
<td>0.128</td>
</tr>
<tr>
<td>*01:04/*01:04</td>
<td>14.3 (4/28)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>*01:01/*01:06</td>
<td>10.7 (3/28)</td>
<td>23.5 (5/21)</td>
<td></td>
</tr>
<tr>
<td>*01:03/*01:04</td>
<td>7.1 (2/28)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>*01:01/*01:03</td>
<td>3.6 (1/28)</td>
<td>9.5 (2/21)</td>
<td></td>
</tr>
<tr>
<td>*01:03/*01:06</td>
<td>3.6 (1/28)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>*01:06/*01:06</td>
<td>–</td>
<td>9.5 (2/21)</td>
<td></td>
</tr>
</tbody>
</table>

The study group consisted of 28 miscarriage samples and the control group included 21 blood samples from healthy individuals. There were 8 HLA-G genotypes. The same genotype name was used for genotypes specific to the same protein, e.g., G*01:04:01/*01:04:04 was shown as *01:04/*01:04.

Table 3. Frequency distribution of the 3’ UTR 14bp ins/del polymorphism (rs371194629) in miscarriage and control samples

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Frequencies in miscarriage samples, % (n)</th>
<th>Frequencies in control samples, % (n)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insertion</td>
<td>43 (24/56)</td>
<td>54 (108/202)</td>
<td>0.301</td>
</tr>
<tr>
<td>Deletion</td>
<td>57 (32/56)</td>
<td>46 (94/202)</td>
<td>0.082</td>
</tr>
</tbody>
</table>

Genotypes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Frequencies in miscarriage samples, % (n)</th>
<th>Frequencies in control samples, % (n)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous insertion</td>
<td>25 (7/28)</td>
<td>27 (27/101)</td>
<td>0.215</td>
</tr>
<tr>
<td>Homozygous deletion</td>
<td>39 (11/28)</td>
<td>20 (20/101)</td>
<td></td>
</tr>
</tbody>
</table>

The study group consisted of 28 miscarriage samples (56 alleles) and the control group included 101 blood samples (202 alleles) from healthy adults.

Discussion

The β2m-associated/free HLA-G dimers are shown to be expressed from trophoblasts. HLA-G stimulates trophoblastic invasion. In addition to this, it is one of the major tolerogenic molecules in pregnancy. The HLA-G expression is studied in preeclampsia, recurrent miscarriage and IVF. The results indicated an association between an HLA-G expression pattern and a risk of RM as well as other disorders.

We proposed that the fetal HLA-G alleles may underlie miscarriage and that the condition may recur, so we could find the miscarriage-prone HLA-G alleles in the conceptus material of mothers with a history of miscarriage. Healthy adults were used as controls and they were assumed not to carry miscarriage-prone alleles. We investigated samples of 204 miscarriage cases that had at least 1 previous miscarriage. Triploidy (9 cases), trisomy 15 (8 cases), trisomy 16 (6 cases), and 45,X (5 cases) were the leading causes of miscarriage in our series, and all cases with genetic pathology were excluded from the study. Twenty-eight out of 204 samples from distinct mothers constituted our study group.

There were 10 HLA-G types coded for 4 specific proteins (Table 1) and 8 genotypes were determined in all groups (Table 2). The G*01:04 allele was found in a homozygous state in 4 cases of the study group (14.3%) and in none of the controls. There was a significant correlation between the fetal HLA-G allele G*01:04 and miscarriage (p = 0.007). As an interesting point, all the samples with the G*01:04 allele also had the 14bp deletion allele. The G*01:04 allele may be linked with the 14bp deletion and had to be investigated in our population.

Ober et al. showed that a variation in the parental HLA-G promoter influences miscarriage rates. They hypothesized that the transmission of a high-risk allele from either parent to the fetus would be associated with fetal loss. Maternal homozygosity for 14bp insertion polymorphism has been proposed to predispose to miscarriage. We found that the allele with the 3’ UTR 14bp deletion polymorphism was the most frequent allele in the study group (57%). The homozygous genotype of the same allele was about 2 times more common in the study group than in the controls (39% vs 20%). The HLA-G allele with insertion was the most frequent (54%) in the control group (Table 3). The 14bp deletion of 3’ UTR seems to be related with miscarriage, but the restricted number of study group subjects (n = 28) unfortunately prevented any statistical association to be revealed.

In conclusion, fetal G*01:04 HLA-G type and the 3’ UTR 14bp deletion polymorphism may underlie miscarriages, and investigations of HLA-G alleles with a larger sample of miscarriage products and related immune components (such as LIRB1 and NKs) may contribute to a better understanding of the miscarriage process.

References


