ORIGINAL ARTICLE

HLA-G 14 bp INSERTION/ DELETION POLYMORPHISM IN RECURRENT PREGNANCY LOSS AMONG A SAMPLE OF IRAQI WOMEN.

Dept.of biotechnology, College of science, Baghdad University.

Abstract

HLA-G displays immunotolerant properties and hence plays important roles in the maintenance of a successful pregnancy and maternal tolerance of the semiallogeneic fetus. HLA-G gene Polymorphism may potentially affect the biological properties of the protein, and a 14-bp insertion/deletion polymorphism in exon 8 of the 3 untranslated region (3 UTR) of the HLA-G gene is thought to influence HLA-G expression. To study the association of the 14-bp insertion/deletion (INDEL) polymorphism with the risk of recurrent pregnancy loss (RPL), polymerase chain reaction (PCR) amplification and genotyping were enrolled on 100 women in the case group (women who have had three or more unexplained RPL) and 100 women in the control group (women who have had at least two normal pregnancy). Our results showed that the frequencies of the +14 bp/+14 bp genotypes were not observed in women with RPL, while that of the +14 bp/-14 and -14 bp/-14 bp genotype was significantly increased in RPL compared with the control group of normal fertile women, in addition a significant differences in the allele frequencies of the HLA-G 14-bp polymorphism were observed. These results suggest a possible significance of the HLA-G 14-bp INDEL polymorphism in the outcome of pregnancy. However, further studies on other polymorphic sites in the 3 UTR and 5 UTR regions, as well as monitoring the serum HLA-G concentration are necessary in order to determine the potential implications of this marker in our population.

Introduction:

The human leukocyte antigen-G gene (HLA-G) is located on the short arm of chromosome 6 within the HLA region. It consists of 7 intorns and 8 exons that code for the heavy chain of the HLA-G molecule. Exons 7 and 8 are always absent in the mature mRNA because of the presence of a stop codon in exon 6 (1). Seven expressed isoforms have been described, 4 of which (HLA-G1-G4) are membranous and 3 (HLA-G5-G7) of which are soluble molecules (2). The proteolytic cleavage of the HLA-G1 isoform generates the soluble HLA-G1 form (3). Compared to the classical HLA molecules, the HLA-G gene contains a modest 46 polymorphisms that map to either the coding or non-coding regions. At the protein expression level, only 15 variants have been reported (4). The polymorphisms mapped to the non-coding regions, particularly those in the 5' upstream regulator region (5' UTR) and 3' untranslated regions (3' UTR), reportedly influence the function of HLA-G molecules and have been implicated in some pathologies such as

Corresponding Author: Hasan F.Al-azzawie.
Address:- Dept.of biotechnology, College of science, Baghdad University.
infertility, preeclampsia, failure in in vitro fertilization, and RPL (1,5). Several studies have demonstrated the importance of the 3' UTR in the HLA-G expression profile (6,7). This region contains several regulatory elements, including a poly-A signal and AU-rich motifs involved in maintaining mRNA stability and isoform alternative splicing patterns, which may influence the function of HLA-G, particularly during pregnancy (8,9). The 14-base pair (bp) (5'-ATTGTTCTATGCCT-3') insertion/deletion (indel) polymorphism mapped to position 3741 in the 3' UTR of exon 8 has gained interest (6). Thus, several reports have indicated that this indel polymorphism is related to HLA-G mRNA stability and splicing patterns involved in generating HLA-G isoforms (6,9,10). Moreover, the 14-bp insertion allele was reported to be associated with low levels of both HLA-G mRNA and circulating soluble HLA-G (sHLA-G) isoforms (10,11). It was also reported that plasma levels of sHLA-G were dramatically lower with the genotype +14-bp/+14-bp than with +14-bp/-14-bp and -14-bp/-14-bp genotypes (7,10). Thus, based on the results of several studies, the HLA-G molecule is considered to be a key player during early and mid-term pregnancy by contributing to the maintenance of gestation throughout pregnancy (8,12,13).

RPL was initially defined as death of the fetus before 24 weeks of gestation at least 2 consecutive times (14). It occurs in 2-4% of reproductive-aged women, of which 40-55% cases remain unexplained (15,16). RSA is one of the most common complications associated with early pregnancy and remains a challenge in gynecology (17). Different potential etiologic factors have been implicated in this condition, such as endocrine regulation, autoimmune reaction, thrombophilia, environmental, psychological, and genetic background, and viral infections (15,18,19).

The interface between the fetus and mother may contribute to the development and maintenance of the pregnant uterus as an immune-privileged site. In fact, the immunologic relationship between the mother and the fetus is determined by fetal antigens and the maternal immune system. Inadequate recognition of fetal antigens may result in failed pregnancy (20,21). Since its discovery, the crucial role of the non-classical HLA-G molecule in pregnancy success has been demonstrated (22,23). Various studies have reported that HLA-G molecules are responsible for maintaining the immune-regulated and tolerogenic environment during pregnancy (8,24,25). Indeed, these molecules are predominately expressed on extra villous cytотrophoblasts, maintaining the immune privileged site. In fact, the immunologic relationship between the mother and the fetus is determined by fetal antigens and the maternal immune system. Inadequate recognition of fetal antigens may result in failed pregnancy (20,21). Since its discovery, the crucial role of the non-classical HLA-G molecule in pregnancy success has been demonstrated (22,23). Various studies have reported that HLA-G molecules are responsible for maintaining the immune-regulated and tolerogenic environment during pregnancy (8,24,25). Indeed, these molecules are predominately expressed on extra villous cytотrophoblasts, maintaining the immune privileged site.

In this study, we examined the association between successful and unsuccessful pregnancies and the HLA-G 14-bp insertion/deletion polymorphism using 2 groups of women: an RPL group and a normal, fertile control group of unrelated women in a Iraqi population.

Material and Methods:-
This study included 100 cases of Iraqi women (mean age 34.18 ± 6.22 years) who had RPL and had consulted the Al-Yarmouk teaching Hospital, Baghdad, Iraq, between April 2014 and June 2015. The control group included 100(mean age 34.67 ± 7.75 years) unrelated, normal fertile Iraqi women with 2 or more uncomplicated pregnancies, without a history of RPL, and with at least 2 live births. Patients with anatomical, endocrine, or metabolic disorders or immunodeficiency and autoimmune diseases were excluded from the study. Ethical approval for the study was obtained from the medical ethics committee of AI-Mustansiryia medicine College /AI-Yarmouk Hospital. All patients and controls provided informed consent and agreed to give blood samples for this case-control study.

Genomic DNA extraction and 14-bp polymorphism genotyping:-
Genomic DNA was extracted from peripheral blood using the Pure gene purification kit (Qiagen; Hilden, Germany) according to the manufacturer protocol. Exon 8 of the HLA-G gene was amplified by polymerase chain reaction (PCR) using the primers GE14HLAG (5'-GTATGCTAAGTAAAGATGC-3') and RHG4 (5'-GGAAGGAATGCTAGTTCA GCATGA-3') (Hvid et al., 1999). The PCR protocol consisted of an initial step of denaturation at 94°C, followed by 35 cycles for 20 s at 94°C, 30 s at 64°C, and 60 s at 72°C, as well as a final extension for 10 min at 72°C. The fragment sizes of the PCR products were analyzed (210/224 bp) based on the presence or absence of a specific band on a 3% agarose gel stained with ethidium bromide and visualized on an ultraviolet transilluminator using a gel documentation system.
Statistical analysis:-
Frequencies of the HLA-G 14-bp indel polymorphism were calculated using the direct counting method. Differences between populations were assessed using a c2 test and Fisher exact test with the SPSS software (ver. 17.0; SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium was evaluated for these polymorphisms within each group using the X^2 test. Statistical significance was defined at the 5% level. We applied Yates correction for continuity and Fisher’s exact test (2-tail) when the sample was small.

Results:-
In this case-control study, we examined the distribution of alleles and genotypes of the most commonly studied HLA-G 14-bp indel polymorphism among women with at least 3 unexplained RSAs and healthy women with at least 2 live births and without a history of RSA. Clinical and demographic characteristics of the RSA and control subjects are reported in Table 1. We observed that among RSA subjects, the number of spontaneous abortions varied from 3 to 8. The percentage of women with 3, 4, 5, 6, 7, and 8 RSAs were 37.5, 26.56, 17.2, 4.7, 6.25, and 8%, respectively. Within the RSA group, 36% of women had no children (100% abortions), while for the remaining women, the loss rate was greater than 50%.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>29.23±4.76</td>
<td>29.89±5.22</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Body mass index BMI(Kg/m^2)</td>
<td>22.37±4.24</td>
<td>22.78±3.88</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Menarche (years)</td>
<td>13.46±1.62</td>
<td>13.35±1.57</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Irregular menstrual history %</td>
<td>65</td>
<td>15</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>4.37±1.22</td>
<td>2.6±1.38</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Abortion</td>
<td>3.5±0.72</td>
<td>0</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

The role of 14bp INDEL polymorphism in RSA:-
Genotyping for the 14-bp deletion polymorphism was done by electrophoresis. The amplified PCR products were either of 224 or 210 bp depending on the deletion of the 14 bp from exon-8. The PCR product was visualized and scored under Gel documentation - UV trans-illuminator after being stained the gel with ethidium bromide stain. (Figure 1). Differences between the two groups were seen by using X^2 analysis (Hardy –Weinberg equilibrium).

![Figure 1: Detection of 14-base pair (bp) INDEL polymorphism on 3% agarose gel. Gel viewed under Gel documentation - UV trans-illuminator after being stained with ethidium bromide stain. Lane M: 25/100 bp marker. Lane 1. 5-8,11,16: Heterozygote for deletion. Lane 2-4,9,10,12-15: Homozygote for deletion.](image)

In this case-control study, we examined the distribution of alleles and genotypes of the most commonly studied HLA-G 14-bp ins/del polymorphism located in exon 8 of the HLA-G gene among Iraqi women with at least 3 unexplained RSAs and healthy women with at least 2 live births and without a history of RSA. The genotype distribution of the 14bp polymorphism in URSA and controls were not in Hardy-Weinberg equilibrium (p <0.05); more heterozygote's were observed compared to expectation. This indicates that 14bp HLA-G polymorphism may have some significance in pregnancy outcome and fetal survival.
There are many homozygotes for the -14bp/-14bp sequence (del/del) in RSA compared to fertile control. While homozygote for the +14bp/+14bp sequence (ins/ins) in this study was absent from RSA women and control groups. The distributions of allele and genotype frequencies of HLA-G 14-bp ins/del polymorphisms are reported in table (3-6 A and B). The frequency of 14bp (-14bp/14bp genotype) in URSA was (52.0%), while the frequency of 14bp (-14bp/-14bp genotype) in control group was (27.0%), and the odd ratio was 2.93(95%CI=1.63-5.27) for both groups.

The estimated frequencies of 14bp (+14bp/+14bp) genotype in URSA was (48.0%), while the frequency of 14bp (+14bp/-14bp) genotype in control group was (73.0%), and the odd ratio was 0.34(95%CI=0.19 - 0.61). The significance analysis was similar in both 14bp (-14bp/14bp and +14bp/-14bp) genotypes which was (5x10^-5).The significance of such association was assessed Fisher’s Exact Probability. Such assessment is more preferred, because it allows for correction of probability and it is not affected by small numbers (less than 5). It seems that the chance of successful pregnancy is greater when the mother is heterozygotes (odd ratio of (+14bp/-14bp) was 0.34) rather than homozygotes (odd ratio of (-14bp/-14bp) was 2.93). Maybe this is reflection of the association between the (-14 bp) sequence and altered balance in HLA-G mRNA (thereby possibly protein) isoforms and isoform concentrations. Survival of fetus from heterozygotes mothers, rather than from homozygotes, may be an advantage for the species because of greater variety of MHC haplotype. It may also contribute to avoiding inbreeding.

Table 2 A:-  Allele and genotype frequencies and Hardy-Weinberg equilibrium of 14-bp insertion/deletion polymorphism in unexplained recurrent spontaneous abortion patients and controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>14-bp Insertion/Deletion Polymorphism</th>
<th>HWE</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>URSA patients (No. = 100)</td>
<td>del/del</td>
<td>ins/del</td>
<td>ins/ins</td>
</tr>
<tr>
<td>Observed</td>
<td>No. 52</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>%</td>
<td>52.0%</td>
<td>48.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Expected</td>
<td>No. 57.8</td>
<td>36.5</td>
<td>5.7</td>
</tr>
<tr>
<td>%</td>
<td>57.8%</td>
<td>36.5%</td>
<td>5.7%</td>
</tr>
<tr>
<td>Controls (No. = 100)</td>
<td>Observed</td>
<td>No. 27</td>
<td>73</td>
</tr>
<tr>
<td>%</td>
<td>27.0%</td>
<td>73.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Expected</td>
<td>No. 40.3</td>
<td>46.4</td>
<td>13.3</td>
</tr>
<tr>
<td>%</td>
<td>40.3%</td>
<td>46.4%</td>
<td>13.3%</td>
</tr>
</tbody>
</table>

URSA: Unexplained Recurrent Spontaneous Abortion; HWE: Hardy-Weinberg Equilibrium

Table 2B:-  Statistical analysis of 14-bp insertion/deletion polymorphism in unexplained recurrent spontaneous abortion patients and controls.

<table>
<thead>
<tr>
<th>Groups</th>
<th>HLA-G 14-bp Insertion/Deletion Polymorphism</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotypes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>del/del</td>
<td>ins/del</td>
</tr>
<tr>
<td>Odd Ratio</td>
<td>2.93</td>
<td>0.34</td>
</tr>
<tr>
<td>Etiological or Preventive Fraction</td>
<td>0.34</td>
<td>0.48</td>
</tr>
<tr>
<td>Fisher’s Exact Probability</td>
<td>5 x 10^-4</td>
<td>5 x 10^-4</td>
</tr>
<tr>
<td>95% Confidence interval</td>
<td>1.63  -5.27</td>
<td>0.19  -0.61</td>
</tr>
</tbody>
</table>

Discussion:-
The genotype distribution of the 14bp polymorphism in URSA and controls were not in Hardy-Weinberg equilibrium (p <0.05). To achieve the equilibrium five conditions must be met: Population must be very large, population must be isolated from other populations (no immigration or emigration), no alleles mutations (deletion or insertion), random mating (no inbreeding) and no natural selection (i.e. every individual has an equal chance of survival). If the five conditions are not met then evolution occurs and there is a change in allele frequency in the population and Hardy–Weinberg equilibrium is not present(28). Because the mutation occurred in allele by deletion 14bp in this study and inbreeding (increased recessive alleles) may occurred that led to deviate in Hardy - Weinberg equilibrium and the genotypes distribution were not in this equilibrium.
This result was agreed with (6) that the genotype distributions of the 14bp polymorphism in fertile controls were not in Hardy-Weinberg equilibrium. This 14-bp polymorphism has been associated with HLA-G mRNA isoforms which lack 92 base sequences in the first part of exon-8 (3' UTR) (29). The 14-bp sequence at the beginning of
exon-8 may be responsible for the alternative splicing of the HLA-G transcript. At the time of mRNA processing, this sequence may function as a cryptic branch point sequence for mRNA splicing. The presence of 14 b in the transcript induces alternative splicing of HLA-G mRNA as a result of which surrounding 92 bases are removed from the mature transcript. This mature transcript without 92 bases is stable in nature. However, when 14b is deleted there is retention of 92 bases in the mature transcript and these results into unstable transcript. This way 14-bp deletion/insertion polymorphism may influence both, the HLA-G isomorph’s splicing patterns (9) and HLA-G mRNA stability (13) therefore may be associated with miscarriages. The mRNA stability may be influenced by AU-rich element of 3' UTR. These AU-rich elements consist of one or many copies of AUUUA pentamer and facilitate the degradation of mRNA. It is hypothesized that because the pentameric initial AUUUG of 14b sequence has AU pentamer-like effect (9), it may be involved in deadenylation and subsequent decay of mRNA. The absence of such a motif in the 92b deleted transcript may interfere with the deadenylation and thus may provide better resistance to mRNA degradation.

Since its discovery by Ellis (30), the non-classical HLA-G class I molecule has been considered to be a key effector in the immune tolerance of the fetus in pregnant women (12,13,14). Based on its specific tissue distribution and level of expression in the body, this molecule received attention for its potential role in pregnancy disorders. Compared to classical HLA genes, HLA-G is characterized by a low level of polymorphism (5,9). Correlations between these polymorphisms, particularly in the 5' UTR and 3' UTR and some pregnancy disorders, have been well-documented. Numerous polymorphic sites have been reported in the 3' UTR (2). The most thoroughly examined is the 14-bp INDEL polymorphism that is important in HLA-G expression, mRNA stability, and alternative splicing and is associated with a wide spectrum of diseases, including pregnancy disorders (31). Thus, this polymorphic site is an important candidate for studying pregnancy disorders, particularly those with unidentified common factors such as RSA.

Our results regarding the association study between the 14-bp INDEL polymorphism and RSA in the Iraqi population agree with those reported in other populations such as the Amerindian populations from the Brazilian Amazon, Finnish, Hungarian, Polish, Japanese, Danish, Chinese, and some Indian populations (2,27,32,33). However, in other studies, the association between the HLA-G 14-bp polymorphism and RSA were confirmed in some Indian and Chinese populations (17). However in a previous study in an Indian population, the HLA-G 14-bp polymorphism was not associated with RSA disorder (20). To assess the strength of the association between the HLA-G 14-bp polymorphism and RSA, a meta-analysis based on 14 studies examining the association in different populations were controversial. This analysis confirmed a strong association between the del (-14bp) allele and RSA risk. However, the association between the heterozygote (+14bp/-14bp) and RSA remains controversial (28.)

References:


