THE IMPACT OF 14-BP INSERTION/DELETION POLYMORPHISM IN EXON 8 OF HLA-G AND ITS EXPRESSION BY REAL TIME PCR AND IMMUNOHISTOCHEMICAL METHODS IN UNEXPLAINED MISCARRIAGE IRAQI WOMEN

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ABSTRACT
To study the association of the 14-bp insertion/deletion (INDEL) polymorphism with the risk of RSA, PCR amplification was used. Insertion or deletion of the 14-bp sequence in HLA-G generated PCR products of length 224 or 210 bp, respectively were observed and the three different genotypes (+14 bp/+14 bp, +14 bp/−14 bp, and −14 bp/−14 bp) were distinguishable by 3% agarose gel electrophoresis. Our results showed that the frequencies of the homozygous genotypes (+14 bp/+14 bp) were not observed in women with recurrent abortion. However, the frequency of homozygous genotypes (-14 bp/-14 bp) was significantly increased in women with RSA compared with the normal fertile control. There were a significant differences in allele frequencies of polymorphism between controls and URSA (OR=1.9259, 95%CI=1.1269-2.3309 and P=0.0179). Real time PCR technique was evaluated in this work, using Smart Cycler system, to detect 14bp insertion/deletion polymorphism in exon 8 of 3’untranslated region of the HLA-G gene. The results showed the accumulation of PCR product was monitored by measuring the level of fluorescence. The results revealed there is no progress of PCR with extracted DNA as the template and the PCR product was not observed when detecting 14bp deletion in patients with RSA, indicating that when 14bp was deleted from DNA of HLA-G will cause defect in HLA-G protein and this lead to women’s abortion. Results of Immunohistochemical experiment was observed that HLA-G expression intensity was decreased in the outer layer of trophoblast cells in the placenta tissue in the first trimester of women with recurrent spontaneous abortion comparing with control based on the 4H84 monoclonal antibody (mAb) against HLA-G protein. This was suggesting that RSA is associated with a lack of expression of HLA-G protein by trophoblast cells, so remains possible that the HLA-G protein expressed by trophoblast cells in women with RSA may be functionally defective.

KEYWORDS: To study Results of Immunohistochemical trophoblast defective.

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 introns 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’t 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 infertility, preeclampsia, failure in in vitro fertilization, and RSA.[5,6] 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] gly, the 14-base pair (bp) (5’-ATTTGTTCTACGCT-3’) insertion/deletion (indel) polymorphism mapped to position 3741 in the 3’ UTR of exon 8 has gained interest.[9] 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.[6,8,9] 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.\textsuperscript{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.\textsuperscript{8,12,13}

RPL was initially defined as death of the fetus before 24 weeks of gestation at least 2 consecutive times.\textsuperscript{14} It occurs in 2-4% of reproduce live-aged women, of which 40-55% cases remain unexplained.\textsuperscript{15,16} RSA is one of the most common complications associated with early pregnancy and remains a challenge in gynecology.\textsuperscript{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.\textsuperscript{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.\textsuperscript{20,21} Since its discovery, the crucial role of the non-classical HLA-G molecule in pregnancy success has been demonstrated.\textsuperscript{22,23} Various studies have reported that HLA-G molecules are responsible for maintaining the immune-regulated and tolerogenic environment during pregnancy.\textsuperscript{8,9,20} Indeed, these molecules are predominately expressed on extravillous cytotrophoblasts at the fetal-maternal interface during pregnancy.\textsuperscript{26} Currently, there is convincing experimental support for expression of HLA-G molecules conferring protection against cytolysis mediated by different maternal cytotoxic subpopulations, such as those of the natural killer cells, T lymphocytes, and dendritic cells. Another study indicated that altered expression of HLA-G molecules is associated with RSA.\textsuperscript{27}

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**

**Subjects:** 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 Al-Mustansirya medicine College /Al-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 \((5'\text{-GTGATGGGCTTAAAGTGTCACC-3')}\) and RHG4 \((5'\text{-GGAAAGGAATGCAGTGTTCA-3')}\) according to Hvid (4). 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).

**Real time PCR assay for genotyping of HLA-G 14-bp insertion/deletion polymorphism in exon 8**

The real-time TaqMan PCR assay was done according to Djurisic (28) by using smartycler instrument. The forward primer, HLAG14-forward, was 5-GTG ATG GGC TGT TTA AAG TGT CAC C-3, and the reverse primer, HLAG14-reversed, was 5-GGA AGG AAT GCA GTT CAG CAT GA-3. The probe used for detection of the 14-bp deletion allele was 5-Cy5-GAG TGG CAA GTCT CCT TTG TG-BHQ-3 (HLAGdelCY5) and the probe for the 14-bp insertion allele was 5-Fam-CAA GAT TTGTTC ATG CCT TCC C-BHQ-1 (HLAG14FAM). Amplification was performed in a 20-μl reaction mixture. The master mixtures were prepared using the following reaction components to the indicated end concentrations: 1μl D.D.W ,1 μl forward primer(10 pmole), 1 μl reverse primer (10 pmole), 1μl HLAG14FAM probe (5 pmole) and 1μl HLAGdelCY5 probe (10 pmole). Then, 15 μl PCR master mix was used to fill each well of a PCR microtiter plate and 5 μl genomic DNA was added as PCR template. The PCR microtiter plates were centrifuged at 3000 rpm for 2 min in a swing rotor to remove small air bubbles in the vessels. The following experimental run protocol was performed according to Djurisic.\textsuperscript{28}

The Immunostaining method was done according to Abcam protocol, UK which used a biotin free immunoenzymatic antigen detection system (ab80438, Abcam) and the primary antibody is Anti-HLAG antibody [4H84] ab5255, abcam. This part of study was done in histopathological laboratories of medical city in Baghdad. Scoring of the Immunohistochemical staining for placenta tissue collected from URSA and fertile
pregnant women was performed according to that reported by (Hviid et al.,2004) as seen below: Score 0:- negative, no staining was observed in less than 10% of the placental cells. Score +1:- negative, a faint/barely perceptible staining was detected in more than 10% of the cells. Score+2:- weakly positive, a weak to moderate complete staining was observed in 20-50% of the cells.Score+3: strongly positive, a strong complete staining was observed in more than or equal 50%.

RESULTS AND DISCUSSION
In the present work, real time PCR technique was evaluated, using Smart Cycler system, to detect 14bp INDDEL polymorphism in exon 8 of 3 untranslated region of the HLA-G gene. The results showed the accumulation of PCR product was monitored by measuring the level of fluorescence intensity. Figure (1) shows the progress of PCR with extracted DNA as the template. The PCR product was observed to accumulate as exponential manner, indicating an optimal PCR. It can be seen that the signals started to rise at different times, because the DNA concentration did not estimate and started with different amounts of DNA of different patients.

![Figure 1](image_url)

Figure 1 The FAM channel of real time PCR for detection 14 – bp INDEL. The colors indicate DNA of different patients, the color indicated patient No. 1; the color indicate the patient No. 5; the color indicate the patient No. 6; the color indicate the patient No.7.

Figure (1) shows there is no progress of PCR with extracted DNA as the template and the PCR product was not observed, indicating that when 14- bp was deleted from DNA of HLA-G will cause defect in HLA-G protein and this lead to women’s abortion. This experiment considers qualitative method for DNA detection, and has several advantages. First, rapid amplification and analysis allow the test to be completed within less than 75 minutes. Because the DNA extraction procedure requires 1 hour, the whole detection can be completed in less than 3 hours. Second, the smart cycler optical device is capable of measuring fluorescence in two separated channels simultaneously (FAM, Cy5), thus allowing of different genotype. Third, the assay is run in closed capillary tubes. Post amplification analysis can be performed without opening the capillaries, minimizing the risk of carry over contamination. Finally, it can be emphasizing that this is the first time that real time PCR has been employed to study the genotype of 14-bp INDEL polymorphism in exon 8 of HLA-G gene in Baghdad city. Reliable results can be obtained faster than with any other current molecular methods.
Figure 2: The Cy5 channel of real time PCR for detection 14-bp deletion in patients with RPM. The colors indicate DNA of different patients, the color □ indicated patient No. 2; the color ■ indicate the patient No. 3; the color ▲ indicate the patient No.4; the color ▼ indicate the patient No.9.

Immunohistochemical study

One of the most characteristic features of human embryonic development is the intimate relationship between the embryo and the mother. To survive and grow during intrauterine life, the fetus must maintain an essentially parasitic relationship with the body of the mother to acquire oxygen and nutrients and eliminate waste products.\(^{[29]}\) The fetus displaying antigens inherited from the father on its cell surfaces must avoid being rejected as a foreign body by the immune system of its maternal host. Reprogramming of immune cell functions into suppressive modes is believed to be a central feature of maternal tolerance to the semiallogenic fetus. A key role in maternal tolerance to the fetus has been attributed to the expression of nonclassical class I MHC molecules HLA-G on trophoblast cells. This molecule has garnered a lot of interest due to its ability to confer protection to the semiallogenic fetus from the maternal immune system, creating a general state of tolerance via interaction with inhibitory receptors presented in maternal killer (NK) cells, T cells and antigen presenting cells (5). HLA-G has been shown to bind to the immunoglobulin-like transcript (ILT)-2 and killer inhibitory receptor (KIR)2DL4 on NK cells and may confer protection to outer layer of trophoblasts via these receptors.\(^{[30,31]}\)

The expression of HLA-G has been studied in different lies of investigation. The unique characteristics of both membrane-bound (G1, G2, G3 and G4) and soluble (G5,G6 and G7) isoforms which arise from alternative splicing and very few amino acid polymorphism ,are well studied.\(^{[32-34]}\) The present data suggesting that HLA-G expression intensity was decreased in trophoblast tissue from first trimester recurrent spontaneous abortion (RSA) comparing with control by the use of immunohistochemistry based on the 4H84 mAb (monoclonal antibody) against HLA-G protein. The figure (3A and B) showed chorionic villi which are one of the most important structures of the placenta. They contain fetal capillaries and are the recipients of the nutrients and gases coming from the maternal blood in order to nourish the fetus to a healthy growth. The outer layer of villi is covered by a layer of trophoblastic cells that can be separated into two different layers .the outer layers is made up of syncytiotrophoblast and the inner layer is made up of cytotrophoblastic cells which are the stem cells of the trophoblast cells.

The syncytiotrophoblast cells form a uniform layer around the villi and in direct contact with maternal blood. In this study there was a difference in the intensity of HLA-G staining by outer layer of trophoblast cells between women with normal pregnancies and with RSA. This result is consistent with the findings of Emmer.\(^{[35]}\) who observed a lower intensity of HLA-G staining of decidual trophoblast cells from women with RSA compared with those from women with normal pregnancies, but this result is contrary study with Patel et al (2003) which found no difference in the expression of HLA-G between women with RSA and normal pregnancies.
Figure 3: photomicrograph depicting the immunohistochemistry of HLA-G in the placenta. (A and B x 400) represent the control (women with known causes of abortion) and the arrows indicated the intensity of HLA-G with score (+3) in the outer layer of trophoblast cells. C and D 400x represent women with RSA and the arrows indicated the intensity of HLA-G with score (+1) in the outer layer of trophoblast cells. E: represent hematoxylin and eosin staining and F show negative control and the arrows indicated there are no appearances of HLA-G.

Peter (36) have shown that in normal pregnancy the expression of the common NK cell antigen CD56 was decreased upon interaction with HLA-G-expressing trophoblast cells. In contrast, they found that a decreased HLA-G expression in trophoblastic tissue obtained from women with a history of recurrent miscarriages was paralleled by an increased expression of both peripheral and uterine NK cell antigens. The results from this study suggest that RSA is caused by a lack of HLA-G expression in the outer layer of trophoblast cells. RSA is associated with a lack of expression of HLA-G protein in trophoblast cells; it remains possible that the HLA-G protein expressed in outer layer of trophoblast cells in women with RSA may be functionally defective.

REFERENCES


