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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 women (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 3́untranslated 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' 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.[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] gly, the 14-base pair (bp) (5'-ATTTGTTCATGCCT-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 reproduce tive-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 extravillous cytotrophoblasts at the fetal-maternal interface during pregnancy.[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.[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-Mustansiryia 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'-GTGATGGGCTGTTTAAAGTGTCACC-3') and RHG4 (5'-GGAAGGAATGCAGTTCA GCATGA-3') according to Hviid (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 smart cycler instrument. The forward primer, HLAG14-forword, 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 CAAGTC CCT TTG TG-BHQ-3-3(HLAGdelCY5) and the probe for the 14-bp insertion allele was 5-Fam-CAA GAT TTGTTC ATG CCT TCC C-BHQ-1-3(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 HLAG14FAMprobe (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.[28]

The Immunostainning 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 INDEL polymorphism in exon 8 of 3́ 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.

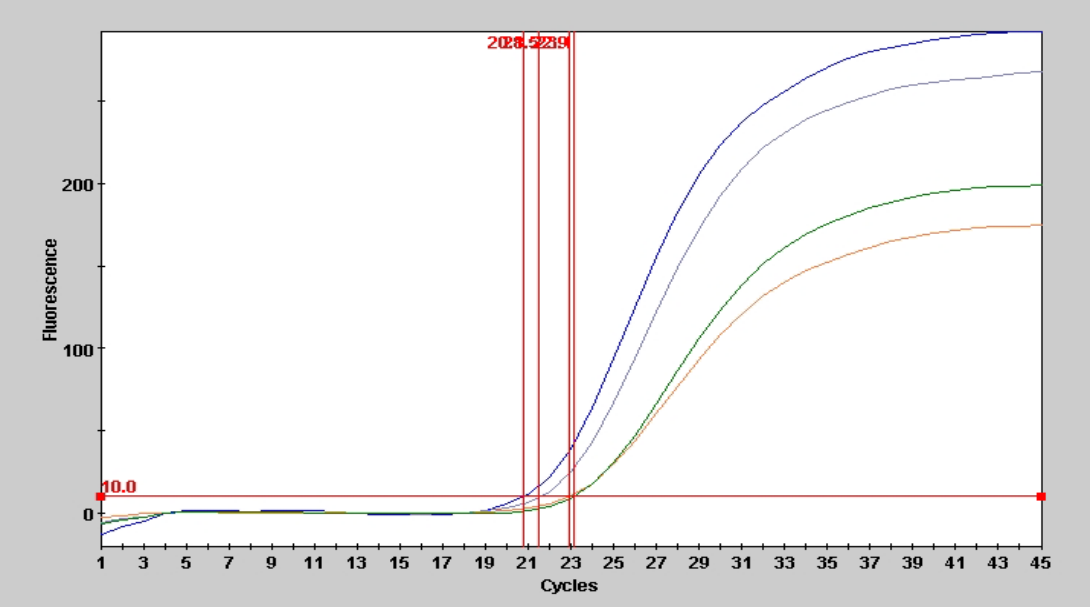


Figure 1 The FAM channel of real time PCR for detection 14 – bp INDEL. The colors indicate DNA of different patients, the color indicated patient No. 1; the color indicate the patient No. 5; the color indicate the patient No. 6; the color indicate the patient No.7.

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 everal 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 florescence 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 opining 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.

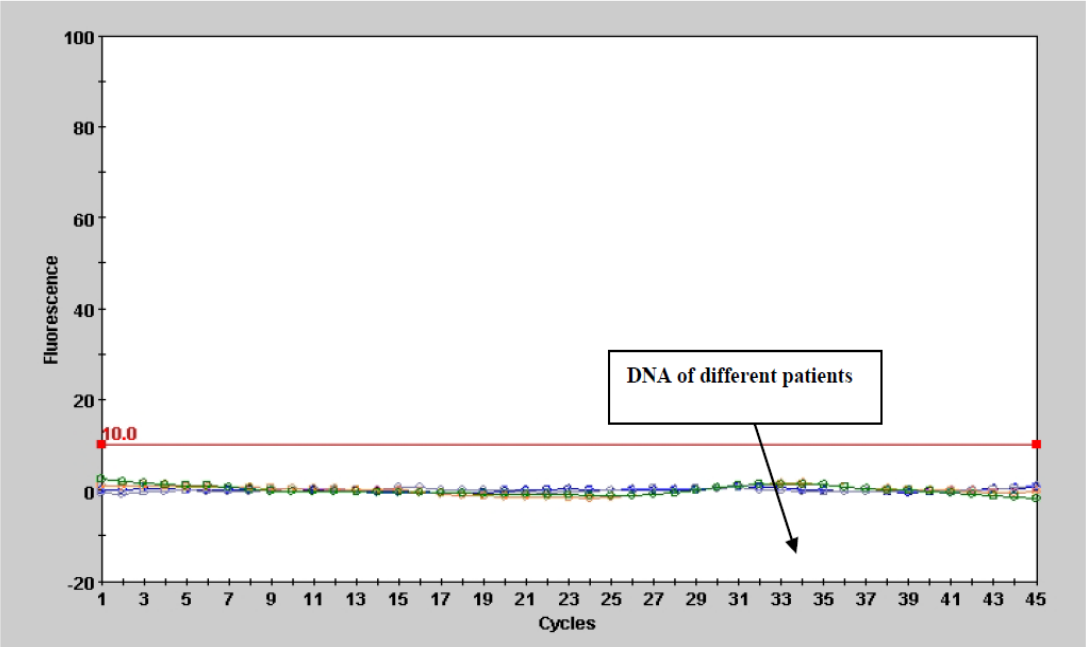


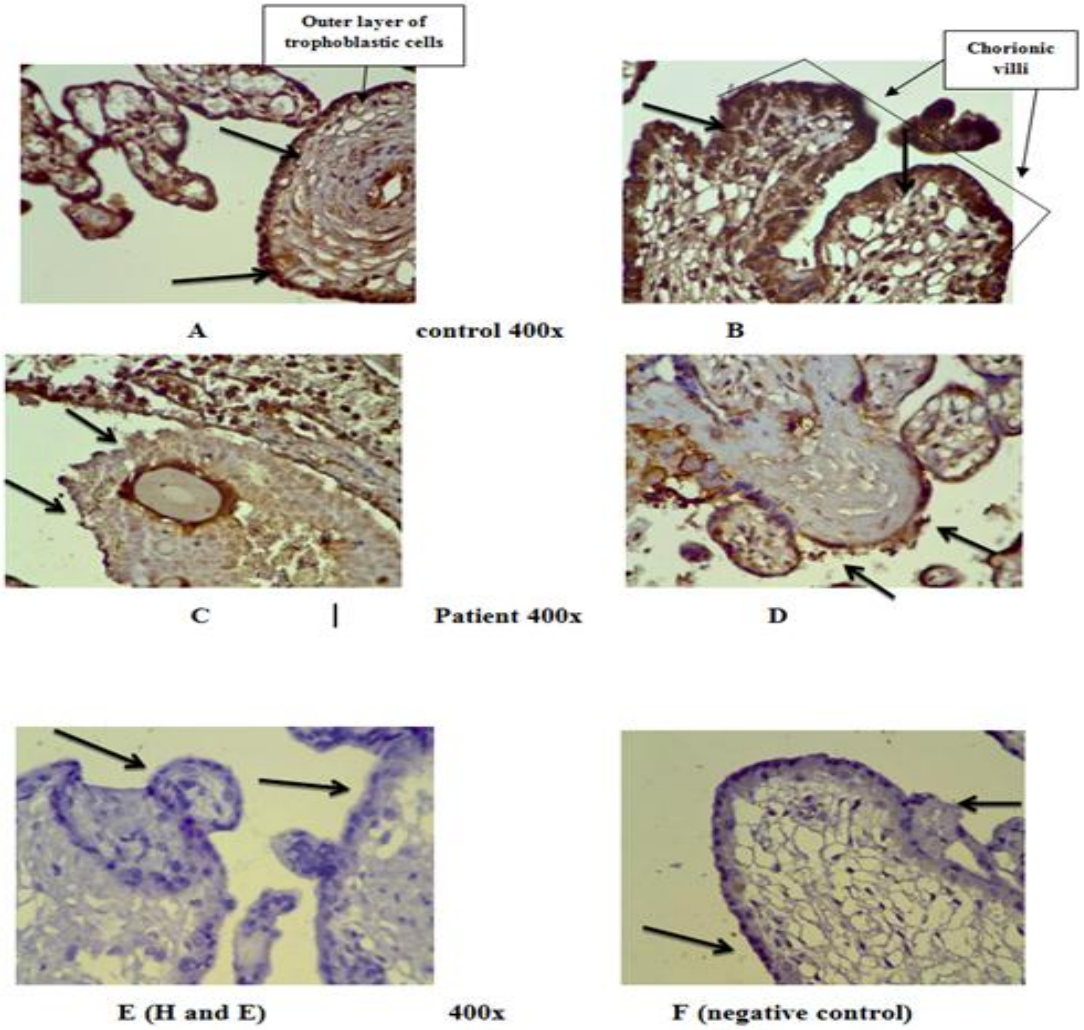
Figure 2: The Cy5 channel of real time PCR for detection 14- bp deletion in patients with RPM. The colors indicate DNA of different patients, the color indicated patient No. 2; the color indicate the patient No. 3; the color indicate the patient No.4; the color indicate the patient No.9.

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 gasses 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 in to 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.



Fgure 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

1. Donadi EA, Castelli EC, Arnaiz-Villena A, Roger M, et al. Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association. Cell. Mol. Life Sci. 2011; 68: 369-395.
2. Rull K, Nagirnaja L, Laan M. Genetics of recurrent miscarriage: challenges, current knowledge, future directions. Front Genet 2011; 3: 34.
3. O'Brien M, McCarthy T, Jenkins D, Paul P, Dausset J, Carosella ED and Moreau P Altered HLA-G transcription in pre-eclampsia is associated with allele specific inheritance: possible role of the HLA-G gene in susceptibility to the disease. Cell Mol Life Sci., 2001; 58: 1943–1949.
4. Hviid TV, Hylenius S, Hoegh AM, Kruse C, et al. HLA-G polymorphisms in couples with recurrent spontaneous abortions. Tissue Antigens 2002; 60: 122-132.
5. Menier C, Rouas-Freiss N, Favier B, LeMaoult J, et al. Recent advances on the non-classical I HLA-G molecule. Tissue Antigens 2010; 75: 201-206.
6. Pfeiffer KA, Fimmers R, Engels G, van der Ven H, van der Ven K. The HLA-G genotype is potentially associated with idiopatic recurrent spontaneous abortion. Mol Hum Reprod 2001; 7(4): 373-8.

7. Martelli-Palomino G, Pancotto JA, Muniz YC, Mendes-Junior CT, et al. Polymorphic sites at the 3' untranslated region of the HLA-G gene are associated with differential hla-g soluble levels in the Brazilian and French population. PLoS One 2013; 8: e71742.
8. Fuzzi B, Rizzo R, Criscuoli L, Noci I, et al. HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. Eur. J. Immunol. 2002; 32: 311-315.
9. Hviid TV, Hylenius S, Rorbye C and Nielsen LG. HLA-G allelic variants are associated with differences in the HLA-G mRNA isoform profile and HLA-G mRNA levels. Immunogenetics. 2003; 55: 63-79.
10. Chen XY, Yan WH, Lin A, Xu HH, et al. The 14-bp deletion polymorphisms in HLA-G gene play an important role in the expression of soluble HLA-G in plasma. Tissue Antigens, 2008; 72: 335-341.
11. Boukouaci W, Busson M, Fortier C, Amokrane K, et al. Association of HLA-G low expressor genotype with severe acute graft-versus-host disease after sibling bone marrow transplantation. Front. Immunol. 2011; 2: 74.
12. Kovats S, Main EK, Librach C, Stubblebine M, et al. A class I antigen, HLA-G, expressed in human trophoblasts. Science, 1990; 248: 220-223.
13. Rouas-Freiss N, Marchal RE, Kirszenbaum M, Dausset J and Carosella ED The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors? Proc Natl Acad Sci USA, 1997; 94: 5249–5254.
14. Rico-Rosillo MG, Vega-Robledo GB. Immunological mechanisms involved in pregnancy. Ginecol Obstet Mex, 2012; 80(5): 332-40.
15. Li TC, Makris M, Tomsu M, Tuckerman E, et al. Recurrent miscarriage: aetiology, management and prognosis. Hum. Reprod. Update. 2002; 8: 463-481.
16. van der Meer A, Lukassen HG, van Lierop MJ, Wijnands F, Braat DD and Joosten I Membrane-bound HLA-G activates proliferation and interferon-gamma production by uterine natural killer cells. Mol Hum Reprod, 2004; 10: 189–195.
17. Shankarkumar U, Shankarkumar A, Chedda Z, Ghosh K.Role of 14-bp deletion /insertion polymorphism in exon 8 of the HLA-G gene in recurrent spontaneous abortion patients. J Hum Reprod Sci, 2011; 4(3): 143-6.
18. Hunt JS, Petroff MG, McIntire RH, Ober C. HLA-G and immune tolerance in pregnancy. FASEB J 2005;
19. Shiroishi M, Tsumoto K, Amano K, Shirakihara Y,Colonna M, Braud VM, et al. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLAG. Proc Natl Acad Sci U S A, 2003; 100(15): 8856-61.
20. Tripathi1 P, Naik S, Agrawal1 S. Role of HLA-G, HLAE and KIR2DL4 in Pregnancy. Int J Hum Gene, 2007; 7(3): 219-33.
21. Haematology AoGaCiOa. HLA-G (major histocompatibility complex, class I, G). 2009.
22. Pistoia VM, F.Ferrone, S. Soluble HLA-G: Are they clinically relevant? Semin Cancer Biol, 2007; 17(6): 469-79.
23. Hunt JS, Pace JL, Morales PJ, Ober C. Immunogenicity of the soluble isoforms of HLA-G. Mol Hum Reprod, 2003; 9(11): 729-35.
24. Martelli-Palomino G, Pancotto JA, Muniz YC, Mendes-Junior CT, Castelli EC, Massaro JD, et al. Polymorphic Sites at the 3' Untranslated Region of the HLA-G Gene Are Associated with Differential hla-g Soluble Levels in the Brazilian and French Population. PLoS One, 2013; 8(10): e71742.
25. Azarpira N, Aghdaie MH, Kazemi K, Geramizadeh B,Darai M. HLA-G Polymorphism (rs16375) and Acute Rejection in Liver Transplant Recipients. Disease Markers, 2014.
26. Harrison GA, Humphrey KE, Jakobsen IB, Cooper DW.A 14 bp deletion polymorphism in the HLA-G gene. Hum Mol Genet, 1993; 2(12): 2200.
27. Yan WH, Lin A, Chen XJ, Dai MZ, Gan LH, Zhou MY,et al. Association of the maternal 14-bp insertion polymorphism in the HLA-G gene in women with recurrent spontaneous abortions. Tissue Antigens, 2006; 68(6): 521-3.
28. Djurisic,s.; sorensen, A., E. and Hviid, T.,V. A fast and easy real- time PCR genotyping method for the HLA-G 14bp insertion /deletion polymorphism in the 3́untranslated region. Tissue Antigens. 2012; 79: 186-189.
29. Morales, P., J.; Pace, J., L.; Platt, J., S.; Phillips, T., A.; Morgan, K.; Fazleabas, A., T., and Hunt, J., S. Placental cell expression of HLA-G2 isoforms is limited to the invasive trophoblast phenotype. J Immunol, 2003; 171: 6215–6224.
30. Allan, D.; Colonna, M.; Lanier, L.; Churakova, T.; Abrams, J.; Ellis, S.; McMichael, A., and Braud, V. Tetrameric complexes of human histocompatibility leukocyte antigen HLA-G bind to peripheral blood myelomonocytic cells. Journal of Experimental Medicine. 1999; 189: 1149–1156.
31. Biassoni, R.; Bottino, C.; Millo, R.; Moretta, L., and Moretta, A. Natural killer cell-mediated recognition of human trophoblast. Seminars in Cancer Biology. 1999; 9: 13–18.
32. Fujii, T.; Ishitani, A. and Geraghty, D. E. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. J. Immunol. 1994; 153(12): 5516-5524.
33. Kirszenbaum, M.; Moreau, P.; Gluckman, E.; Dausset, J. and Carosella, E. An alternatively spliced form of HLA-G mRNA in human trophoblasts and evidence for the presence of HLA-G transcript in adult lymphocytes. Proc. Natl. Acad. Sci U S A. 1994; 91(10): 4209-4213.
34. Hviid, T., V.; Moller, C.; Sorensen, S., and Morling, N. Co-dominant expression of the HLA-G gene and various forms of alternatively spliced HLA-G

mRNA in human first trimester trophoblast. Hum. Immunol. 1998; 59(2): 87-98.
35. Emmer, P.; Steegers, E.; Kerstens, H.; Bulten, J.; Nelen, W.; Boer, K. and Joosten, I. Altered phenotype of HLA-G expressing trophoblast and decidual natural killer cells in pathological pregnancies. Human Reproduction. 2002; 17: 1072–1080.
36. Peter, M., E.; Eric, A., P.; Harold , M., J.; Johan,B., and Willianne, L., D. Altered phynotype of HLA-G expression trophoblast and decidual natural killer cells in pathological pregnancies .Human Reproduction. 2002; 17(4): 1072-1080.