Dissemination of 14bp deletion/insertion gene polymorphism of Human Leukocyte Antigen class I (G) with recurrent Spontaneous abortion in Baghdad

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Abstract. Recurrent spontaneous abortion (RSA) is a surprisingly common occurrence in various populations, risk factors associated with pregnancy losses are largely variable and often changes among different communities. This study was designed to determine the possible association between HLA-G 14bp insertion/deletion gene polymorphism with recurrent spontaneous abortion. Peripheral blood was collected from 210 women (180 women with recurrent abortion three or more abortions and 30 women with normal pregnancy to three or more births and without any previous abortion) in the first trimester. Based on clinical examination and diagnostic laboratory findings of ELISA for TORCH test were selected ninety from 180 women with recurrent abortion in the current study were divided into three groups: group one included 30 women with recurrent abortion with sero-negative for TORCH test, group two also 30 women with recurrent abortion with ser-positive for anti-toxoplasma antibodies, while control group included 30 women with a healthy pregnancy. In the current study not found any significant alteration between heterozygous and homozygous amongst three groups, also not found any implication between recurrent abortion and healthy pregnant in the field of alleles (+14bp insertion or -14bp deletion). The genotyping and alleles of HLA-G 14bp (insertion/deletion) were not give in to the hypothesis of connotation between HLA-G and recurrent spontaneous abortion.

Keywords: Recurrent spontaneous abortion, Toxoplasma, s HLA-G.

Introduction

Recurrent Spontaneous abortion (RSA) is the most communal problem of early pregnancy, refers to one of the most common reproductive disease. It is well-defined as (three or more) recurrent pregnancy losses earlier the fetus has not reached a viable gestational age. The most common causes of miscarriage include genetics, immunologic factors, endocrine disorder, and infection with microorganisms like Toxoplasma gondii, Cytomegalovirus, and maternal disease such as thyroid disease and diabetes mellitus[1]. Toxoplasmosis caused abortions typically occur through the first partial of pregnancy. It might lead to acute harm or abortion. Embryo performances as an allograft to
the mother’s body, it is persisting normally in the mother’s uterus through the complete gestational period in the event of normal successful pregnancy [2].

Human Leukocyte Antigen-G (HLA-G) is non-classical Class I major histocompatibility complex [3], can performance a critical role in regulating CD8+ T cells through pregnancy by removing all reactive anti-paternal T cell [4]. HLA-G protein potentially exists as seven isoforms including four membrane-bound HLA-G1, HLA-G2, HLA-G3, and HLA-G4 as well as three secreted soluble HLA-G5, HLA-G6, and HLA-G7 proteins [5]. HLA-G gene is located on the short arm of the chromosome 6. It is (4170) bp long and consists of 8 exons and 7 introns that code for the heavy chain of the HLA-G molecule [6]. Exons 8 and 7 are always absent in the mature mRNA because of the presence of a stop codon in exon 6, HLA-G gene contains a modest 46 polymorphisms that map to either the coding or non-coding regions [5].

The aim was to examine if there is association of HLA-G with spontaneous abortion and detection if there is correlation of the 14bp deletion/insertion polymorphism of HLA-G gene with recurrent abortion and to investigate whether gene polymorphisms and alleles for the HLA-G could be used as markers of susceptibility to pregnancy loss

Materials and Methods

Patient and control

The recent study included 180 women with recurrent abortion during the first-trimester and 30 women as a control group were with normal third delivery or more and with no previously recognized miscarriage. The ages of these women were ranged between 20-30 years.

The total numbers of women were 210 collected from different Hospital in Baghdad during the period from March to December in 2016.

Five milliliter of venous blood was taken at the time of miscarriage by using sterile disposable syringes, (3ml) of blood placed in a plain tube with gel clot and left to stand for one hour at room temperature for clot formation, for serum collection, the tube centrifuged for 10 minutes at 3000 revolution per minute (rpm). Then the serum aspirated by using a Pasteur pipette and dispensed into sterile Eppendorf tube and stored at -20°C until used. While 2ml of blood samples were transferred to di-potassium ethylene diamine tetra acetic acid (K2EDTA) tubes and incubated in the Refrigerator for extraction of DNA.

According to TORCH of ELISA test were divided into

- **Group one:** Thirty women with repeated aborted three or more during the first trimester with sero-negative for TORCH test (NO= 50). Selected for this study 30 cases only (group1).

- **Group two:** Thirty women with repeated aborted three or more during the first trimester with sero-positive for anti-toxoplasma antibodies (NO= 44). Selected for this study 30 cases only (group2).

- **Group three:** Women with repeated abortion three or more during the first trimester with seropositive for one or more causes of TORCH test such as infection with *Herpes virus, Rubella virus and Cytomegalovirus* (NO= 86). But not selected in the study, out of procedure.

- **Group of control**

  Pregnant women with normal third delivery or more during the first trimester and with no previously recognized miscarriage (NO= 30).
Genetic variation study

DNA extraction

DNA isolation genomic DNA was extracted from the peripheral blood samples using the salting-out procedure\(^7\).

Determination of DNA Concentration and purity

The concentration and purity of DNA was measured by Quantus Fluorometer.

Primers

PCR reaction was performed by using a specific primer pairs designed for HLA-G gene. Based on NCBI database, all gene information, sequence and SNPs details, were collected. By using genius software. The sequences of this primer were explained in the table (1).

| Table (1): Primer Sequence used for this gene. |
|------------------------------------------------|
| **Gene** | **Primer Sequence** |
|----------|---------------------|
| 14(ins/del)F | 5'-TGATGTGTGTTGAGGG-3' |
| Primer with M13 Tail | 5'-TGAAAACGACCCAGTTGATGTTGGTTGGAGGG-3' |
| 14(ins/del)R | 3'-ACAAGAAACACGTGTACTGTGGAAA-5' |
| Primer with M13 Tail | 3'-CAGGAAACAGCTATGACCACAAGAAACACGTGTACTGTGGAAA-5' |

Amplification of DNA by PCR

The HLA-G gene is sited on the short arm of the chromosome 6. It is 4170 base pair (bp) long and consists of 7 introns and 8 exons. The 14bp (5'-ATTGTTCATGCCT-3') insertion/deletion polymorphism mapped to the non-coding regions, location 3741 in the 3’ untranslated region (3’ UTR) of exon 8. For analyzing the HLA-G of (14bp) Insertion/Deletion Polymorphism among patient and control, PCR amplification of exon 8 of the HLA-G gene was completed for all cases by using a specific primer pair that planned for this project. Sanger sequencing were done for all PCR products.

PCR program.

In this reaction, specific temperature for HLA-G gene, the reaction was carried out as follows in the table (2):

| Table (2): PCR program for PCR amplification |
|---------------------------------------------|
| **Steps** | **C°** | **min:** | **Cycles** |
| Initial Denaturation | 95 C° | 5 min | 1 |
| Denaturation | 95 C° | 30 sec | 35 |
| Annealing | 60 C° | 30 sec | 35 |
| Extension | 72 C° | 30 sec | 35 |
| Final Extension 2 | 72 C° | 7 min | 1 |
| Holding | 4 C° | -- | 1 |
Agarose Gel Electrophoresis

After DNA extraction and PCR amplification, it was prepared according to Sambrook method [8].

Standard Sequencing

PCR product were send for sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation-Korea. The results were received by email then analyzed by using genius software.

Statistical analysis

Chi-square test used to significant compare genotype for each sample, Odd ratio and confidence intervals were used to assess the risk or beneficial effect of studied factor between groups [9].

Results

Distribution of 14 base pair (bp) Insertion/ Deletion polymorphism in the study groups

The result of the HLA-G gene polymorphism in recurrent abortion patients and healthy group of HLA-G gene polymorphism were the frequencies of homozygous (insertion) +14bp/+14bp in group1 with recurrent abortion (6), group 2 (8) and group 3 (10) and homozygous (deletion) -14bp/-14bp results were in group1 (8), group 2 (3) and group3 (5) while heterozygous +14bp/-14bp results were in group 1 (16), group 2 (19) and group 3 (15). Revealed statistically no significant differences of HLA-G 14bp deletion/insertion genotype between patient with recurrent abortion and healthy control. As shown in the table (3).

Table (3): Frequencies of the 14 bp genotype in exon 8 of HLA-G gene

| Study groups | Recurrent abortion with sero-negative for TORCH test (group1) n (%) | Recurrent abortion with sero-positive for anti-Toxoplasma antibody (group2) n (%) | Pregnant healthy (Control group) n (%) |
|--------------|--------------------------------------------------------------------|---------------------------------------------------------------------------------|--------------------------------------|
| +14bp / +14bp| 6 (20%)                                                             | 8 (26.7%)                                                                       | 10 (33.3%)                          |
| -14bp / -14bp| 8 (26.7%)                                                           | 3 (10%)                                                                         | 5 (16.7%)                           |
| +14bp / -14bp| 16 (53.3%)                                                          | 19 (63.3%)                                                                      | 15 (50%)                            |
| Total        | 30 (100%)                                                           | 30 (100%)                                                                        | 30 (100%)                           |

1-Between group1 and group3: The chi-square statistic is 1.72. The P-value is 0.42. The result is not significant at p < 0.05.
2-Between group2 and group3: The chi-square statistic is 1.19. The p-value is 0.55. The result is not significant at p < 0.05.

Distribution of 14 bp alleles in the different groups.

The result of the 14 bp alleles in patients and healthy were insertion 14bp in group1 (20), group 2 (19) also, in control group (25) while deletion 14bp were in group1 (40), group 2 (41) and control group (35). The frequencies of the insertion 14bp alleles in control group was higher than in group1,2 and deletion 14bp alleles in group 1,2 was higher than in control group, but not reach to significant level at p < 0.05. as shown in the table (4).
Table (4): Frequencies of the 14bp alleles among different groups

| Study groups | +14bp | -14bp | Total | Odds ratio | confidence interval (95% CI) | P-value |
|--------------|-------|-------|-------|-----------|---------------------------|---------|
| Group 1      | 20    | 40    | 60    | 0.700     | 0.33 - 1.47               | 0.34    |
| Group 2      | 19    | 41    | 60    | 1.00      | 0.30 - 1.37               | 0.64    |
| Control group| 25    | 35    | 60    |           |                           |         |
| Total        | 64    | 116   | 180   |           |                           |         |

1-Between group1 and control group: The chi-square statistic is 0.88. The p-value is 0.34. This result is not significant at p < 0.05.
2-Between group2 and control group: The chi-square statistic is 1.29. The p-value is 0.25. This result is not significant at p < 0.05.

Discussion

The HLA-G gene is located on the short arm of chromosome 6. It is (4170) bp long and consists of 8 exons and 7 introns that code for the heavy chain of the HLA-G molecule \([6]\). The 14bp (5'-ATTGTTCATGCCT-3') insertion/deletion polymorphism mapped to position 3741 in the 3' UTR of exon 8 has gained interest \([10]\). HLA-G molecules are generated by an alternative splicing of the primary transcript of the gene and exhibition specialized function in regulating the immune response. The 14bp insertion/deletion polymorphism influence of HLA-G expression and it has been shown to have a consequential role in post transcriptional regulation of HLA-G molecules \([11, 12]\).

Previous studies have examined the correlation of 14bp polymorphism with the increased risk of recurrent miscarriage \([13, 14]\). However, the studies have provided controversial results which not found correlation of 14bp polymorphism with the increased risk of recurrent miscarriage \([15, 16, 17]\).

Sipak-Szmigiel et al. showed no significant difference between recurrent spontaneous abortion and healthy control in the heterozygous and homozygous of 14 bp gene polymorphism \([18]\). Alomar et al. recorded that the frequency of 14bp (heterozygous and homozygous) of HLA-G genotype was not significant differences in recurrent spontaneous abortions as compared with healthy control \([19]\). Also, many studies found that homozygotes (-14bp/-14bp and +14bp/+14bp) for the 14bp was not associated with recurrent abortion \([20, 21]\).

Durmanova and Drobný in 2016 revealed that no significant differences of HLA-G 14bp deletion/insertion genotypes between women with Preeclampsia and healthy pregnant \([22]\). Also, other studies showed no correlation of 14bp HLA-G genotype with increased risk of abortion, obstetrical complications and Pre-Eclampsia \([23, 24, 25]\). Yan et al. observed that distribution of 14bp insertion/deletion genotype of HLA-G had no significant difference between healthy controls and recurrent spontaneous abortion \([21]\). Also, Pabalan et al. revealed that no associations between the HLA-G 14bp ins/del genotype and development of Pre-Eclampsia in European Caucasian pregnancies \([26]\). Also, other study reported that no correlation between increased number of heterozygous and recurrent abortion \([18]\).

Zhu et al. reported that both the (-14bp/-14bp and +14bp/-14bp) genotype frequencies were not significantly different between recurrent miscarriages (four or more abortions) and healthy women. However, the frequency of (+14bp/+14bp) homozygotes was increased in recurrent abortions compared with healthy women \([27]\).

Several studies indicate that insertion homozygous of (+14bp/+14bp) fragment is associated with reduced mRNA of HLA-G, which in turn decreases the level of HLA-G produced \([28, 14]\). The increase in the frequency of the (-14bp/-14bp) deletion homozygotes and lower frequency of the +14 bp/-14bp
heterozygotes in the recurrent spontaneous abortion women compared with healthy control, could lead to extremely high levels of soluble HLA-G in recurrent spontaneous abortion [29].

However, the frequency of +14 bp/-14 bp heterozygotes polymorphism in exon-8 was significantly higher in recurrent miscarriage patients as compared with fertile controls [30]. Moreau et al. reported that the homozygous for the HLA-G insertion polymorphism (+14bp/+14bp) was higher in women with recurrent spontaneous abortion comparison to women with heterozygous [31]. The plasma concentration of s HLA-G is lower level among the insertion (+14bp/+14bp) homozygous women in comparison with women of the two-other deletion homozygous (-14bp/-14bp) and heterozygous (+14bp/-14bp) HLA-G genotypes [32,33].

Other studies observed that the higher number of heterozygous in recurrent abortion more than in healthy control [34,35,12]. Also, the presence of heterozygote genotype insertion/deletion (+14bp/-14bp) was associated with increased risk of abortion [14]. Afkhami et al. found that the frequencies of homozygous deletion (-14 bp/-14 bp) and insertion (+14 bp/+14 bp) genotypes were reduced in women with recurrent spontaneous abortion, while heterozygous (+14 bp/-14 bp) genotype was increased in recurrent spontaneous abortion compared with the normal fertile women [12]. Shankar Kumar et al. indicated that homozygotes insertion (+14bp/+14bp) and heterozygote (+14bp/-14bp) genotypes in exon 8 of the 3′UTR region of the HLA-G gene were in recurrent spontaneous abortion more than in healthy women [36]. Furthermore, the homozygotes insertion (+14 bp/+14 bp) genotype was found in recurrent spontaneous abortion patients with abortions greater than 3 as observed by [37,33,21].

These various results are perhaps because of difference in the allocation of the polymorphism in different ethnic populations all over the world and potential linkage disequilibrium with other HLA variation, differences in the study design, and classification of recurrent abortion, control groups and examination of only women experiencing recurrent abortion instead of examination of couple or placenta. The various polymorphisms in the HLA-G gene might vary the mRNA profile expression and the type of protein formed, HLA-G encodes 7 protein isoforms generated by alternative splicing [38].

Several studies show that specific types of HLA-G alleles are associated with increased risk of recurrent spontaneous abortion [39].

Afkhami et al. found that no significant differences in the allele frequencies of the HLA-G 14bp polymorphism in recurrent abortion compared with healthy control [12]. Duranova and Drobot in 2016 found that no significant differences of HLA-G 14 bp deletion or insertion allele between women with Pre-Eclampsia and healthy pregnant [22]. Also, other studies not found any correlation of insertion 14 bp HLA-G alleles with increased risk of abortion, obstetrical complications and Pre-Eclampsia [23,24,25,17].

Alomar et al. observed that the distribution of allele frequencies of 14bp insertion or deletion were not significant differences in the recurrent spontaneous abortion and healthy controls [19]. Also, others studies found no correlation between HLA-G alleles and recurrent spontaneous abortion [15,18] While Wang et al. found that the +14 bp insertion allele of HLA-G is associated with increased risk of unexplained recurrent spontaneous abortions [40]. Also, the frequency of 14bp insertion alleles was increased in recurrent spontaneous abortion compared with healthy controls [33,21].

Shankar Kumar et al. showed that an increase in the frequency of +14bp insertion alleles in the RSA as compared to control pregnant women [41]. The frequency of +14bp insertion allele was significantly higher in recurrent miscarriage as compared with normal fertile controls [42]. The +14 bp allele frequency was highest in women with (four or more) miscarriages, medium in those with two miscarriages, and lowest in fertile controls, although the differences did not reach to the statistical significance [27].
Conclusion

The high serum level of s HLA-G found in recurrent abortion as compared with healthy pregnant and the HLA-G 14bp deletion/insertion polymorphism in exon 8 has no important statistical association with recurrent abortion and healthy pregnant women.

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